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UNDERSTANDING AND IMPROVING CARBOHYDRATE COMPOSITION TO ADD VALUE TO LENTILS

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UNDERSTANDING AND IMPROVING CARBOHYDRATE COMPOSITION TO ADD VALUE TO LENTILS

Saskatchewan Agriculture Development Fund Project # 20040514

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(A-1) EXECUTIVE SUMMARY

The long term objective of this research project is to develop lentil lines with changed carbohydrate composition. The main objectives of this project were: (i) to develop carbohydrate profiles for lentil germplasm currently in use at CDC for lentil improvement program; (ii) to study the genotype x environment interaction ana analyze the heritability of seed carbohydrate related traits; (iii) scan global lentil germplasm to identify the extent of variation in selected carbohydrates available in nature; (iv) to identify and characterize basic biochemical steps for selected carbohydrate biosynthesis. The project was co-funded by the Saskatchewan Agriculture Fund and the Saskatchewan Pulse Growers. The results related to soluble carbohydrates and specifically raffinose family oligosaccharides (RFO) are presented in this report. A separate report including results related to starch and amylose will be submitted to Saskatchewan Pulse Growers by May 31, 2009, the end date for Saskatchewan Pulse Growerspart of the project.

The project was conducted as four major studies, corresponding to the milestones and objectives for the project.

(i) The first objective was to optimize the methods and develop carbohydrate profiles for selected lentil germplasm (22 lines). In addition carbohydrate profiles for six wild lentil species were also developed.

(ii) The second objective dealt with the influence of environment on the accumulation of seed carbohydrates in eleven lentil genotypes grown in ten environments in Saskatchewan. The data was analyzed for genotype and environment and heritability analysis.

(iii) The third objective added by the Review committee was to analyze lentil global germplasm for variation in selected carbohydrates. An ICARDA mini-core collection was obtained and grown at two locations in Saskatchewan during 2006 and 2007. The mini- core collection contained both domesticated and wild lentils.

(iv) The fourth objective was to optimize biochemical assays for determining galactinol synthase (GS) enzyme activity. GS activity was also determined in lentil genotypes with varying amount of total soluble sugars (RFO).

The first three objectives were completed and the work in the fourth objective is still continuing. This was the first project of its kind to conduct systematic analysis of carbohydrates in lentils. The results obtained were used to prepare a five year project to achieve the long term objectives of improve lentil carbohydrate profiles for the benefit of Saskatchewan producers. The project was submitted to Natural Science and Engineering Research Council (NSERC) for a cooperative research and development (CRD) project with Saskatchewan Pulse Growers as industrial partners. NSERC approved the project (C$534,760)

in December 2008. The start date for the CRD project is March 1, 2009 for a period of five years (2009-2014) to end on February 28, 2014.

(A.2) ABSTRACT/SUMMARY

Pulse crops which include peas, lentils, chickpeas and beans have emerged as a major growth area in Saskatchewan agriculture during the eighties. The nutritive and health benefits of pulses in the human diet are well known. While the largest profit margins exist in crops directed towards human consumption, significant markets also exist in animal feed. With increased awareness of climate change, there is increased interest in the beneficial environmental impact of using pulse crop rotation to increase the nitrogen content of soil, thereby reducing the use of chemical fertilizers. In order to remain competitive in these markets, it is paramount to be able to meet changing consumer demands. One area for improving the quality of pulses lies in the fact that they can cause flatulence and abdominal discomfort in both humans and other mono-gastric animals. This is due to the relatively high levels of the raffinose family of oligosaccharides (RFOs) found in pulses.

The major objective of this research was to develop strategies for developing lentils with reduced RFO concentration. In this project we first developed carbohydrate profiles (starch, amylose, total soluble sugars, glucose, sucrose, RFO, raffinose, stachyose and verbascose) for selected lentil germplasm currently in use for lentil improvement programs in Saskatchewan. The results showed that domesticated lentil germplasm had limited variation in carbohydrate concentration. However, higher variation in carbohydrates was found in the wild lentil accessions studied. The second study was conducted to study the influence of environment on RFO accumulation in eleven lentil genotypes. Results showed that environment influenced RFO accumulation in lentil cultivars. Results also showed that RFO concentration was a highly heritable trait and amenable to genetic improvement by standard plant breeding techniques. Scanning a subset of global lentil germplasm collection showed that wild lentils show more diverse range of RFO concentration and composition, confirming the results of objective 1. The results suggest that variation in lentil RFO concentration is available among the pool of lentil germplasm available in gene banks. Therefore, lentil RFO can be reduced using standard plant breeding techniques. However, to accelerate the development of reduced RFO lentil varieties, molecular strategies need to be developed.

(b) INTRODUCTION, BACKGROUND AND OBJECTIVES

Grain Legumes

Grain legume crops, such as pea, lentil, chickpea and bean, are important field crops, due to the nitrogen fixing ability of the growing plant which enriches soil nitrogen and the seed protein rich in essential amino acids that are deficient in cereal grains. Lentil (*Lens culinaris* Medikus) is an important pulse crop, whose cultivation in Western Canada rapidly expanded during the last two decades of twentieth century. More than seventy percent of the lentil crop in Western Canada is exported, especially in Saskatchewan, a majority of lentils produced are exported. The nutritional value of lentils has gained considerable interest in the last few years and programs to improve pulse quality have been initiated. The proximate composition of lentils has been studied mostly with an emphasis on protein and only in a relatively limited number of cultivars (Wang and Daun 2006, Petterson et al., 1997; Sosulski et al., 1976).

Lentils protein is of interest because like most legumes it is rich in lysine and deficient in the sulphur containing amino acids and tryptophan. Most cereal grains are deficient in lysine and adequate amounts of methionine and cysteine. Therefore, lentils are a good supplement in diets rich in cereals. Additionally the lentil protein content is much higher than that in most cereal grains. Wang et al studied the protein content of lentils grown in western Canada between 1998 and 2003 and found a range from 25.8 to 27.1%. They found a large variation in protein content within the same variety due to general environmental effects like temperature and rainfall as well as due to partially controlled agronomic practices (Wang and Duan, 2006).

Grain carbohydrates definitions and distribution

Carbohydrates in grains are classified based on their chemical structures and on their digestibility when consumed by humans as food or by livestock as feed. Based on the polymeric nature of carbohydrates, they are classified as monosaccharides, disaccharides, oligosaccharides and polysaccharides (Chibbar et al., 2004). Monosaccharides are the simplest of all sugars and are the building blocks of all larger carbohydrates. The most common monosaccharides in grains are the hexoses (fructose, glucose and galactose) and the pentoses (arabinoses and xylose). Sucrose and maltose are the most commonly occurring disaccharides in most grains. Oligosaccharides include the sugars that contain three to twenty sugar molecules. Legume crops in general have a higher concentration of di- and oligosaccharides as compared to cereal grains. Especially, chickpeas have a high concentration of soluble sugars, of which sucrose is the major component. Raffinose, stachyose, verbascose and α-galactosides are the most common oligosaccharides in legumes and constitute 6-18% of the seed dry weight. Lentils and peas have higher concentrations of stachyose and verbascose as compared to other legumes. Polysaccharides are polymers with more than twenty monosaccharides. These are complex molecules because of the diversity in monosaccharide units and type of linkages present in

the polysaccharides. Some of the most common polysaccharides present in grains are starch, cellulose and xylans, of which xylans include pentosans, (â-glucans, and arabinoxylans). Legume seeds contain about 50 percent starch, except lupins and soybeans in which only 0.5 to 1.4% of the dry weight is starch. The major non-starch complex carbohydrate in grain legumes is cellulose, which represents about one-eighth of the grain dry weight in the majority of the grain legumes.

In relation to human and animal nutrition, carbohydrates are also classified as "available" and "unavailable". The available carbohydrates, represented by sugars and starches, are easily broken down and absorbed by the human and animal digestive tract to give energy to the body. Structural cell wall carbohydrates such as cellulose, hemicellulose and non-starch complex carbohydrates such as xylans are categorized as unavailable carbohydrates because they are not assimilated by the digestive tract. The unavailable carbohydrates are also referred to as dietary fibers.

Starch

Lentil starch is of interest because of its properties and nutritional value that are in a large part determined by the amylose to amylopectin ratio. The physicochemical properties among cultivars have been studied (Hoover and Manuel (1995),Hoover and Ratnayake, 2002). Little is known about the nutritional properties of lentil starch. Pulse starch in general tends to be higher in amylose compared to cereal grain starches. Among pulses lentil and chickpea starch has been reported to contain more amylose compared to other pulse starches (Chibbar et al. 2004). Elevated amylose increases the proportion of re-crystallized starch after cooking or processing at higher temperature and cooling, a process called retrogradation. This starch is often resistant to pancreatic amylase action and is called resistant starch, to be more precise resistant starch type 3. Amylose content has been associated to the amount of resistant starch produced in foods.

Resistant starch is not digested and absorbed in the stomach and small intestine and causes minimal increase in the post-prandial glucose potential and so has a relatively low glycemic index (the blood glucose raising potential) relative to other grain starches. Lentils and lentil starch could become desired in the diets of people with type-2 (insulin dependent) diabetes as well as obesity. Other potential interest areas for resistant starch utilization include the prevention of cardiovascular disease, diarrhea and boosting the immune status by lowering colon pH after fermentation by the microflora in the large intestine.

Oligosaccharides

The carbohydrates of lentils include mono -, di- and oligo-saccharides, starch and other polysaccharides. Oligosaccharides (OS) are carbohydrates with DP (degree of polymerization) 3 to 20. Oligosaccharides of the raffinose family include raffinose, stachyose, verbascose, ajugose and other higher homologes.

Raffinose family oligosaccharides (RFOs) or α – galactosides are sucrosyl galactosides in which the galactose residues are linked by an α-1,6 glycosidic bond to the C-6 of the glucose moiety of sucrose. They are found in seeds, roots and tubers of many plant species (Avigad and Dey, 1997). Oligosaccharides of the raffinose family accumulate in the seeds of many plant species where they act as a source of energy in seed germination and also play a role in the dessication and cold tolerance of seeds. The concentration of these oligosaccharides varies in different species but can make up to two to ten percent of the seed dry weight (Guillon and Champ, 2002).

Humans and other monogastric animals are deficient in the alpha-galactosidase enzyme required to break down the alpha (1-6) linkages between the galactose residues in these oligosaccharides. As a result, RFO pass undigested through the stomach and lower intestine and are fermented in the colon by anaerobic bacteria. Both positive and negative effects on human health have been attributed to the products of fermentation of these oligosaccharides. The products of fermentation include short chain fatty acids (SCFA) mainly acetic, propionic, butyric acid, hydrogen, carbon dioxide and methane (Ruppin et al., 1980). The amount and type of these products of fermentation depends on the type of carbohydrate. Raffinose, barley *b*-glucans, oligofructose and some resistant starch are reported to produce butyric acid in vitro (Casterline et al., 1997; Karppinen et al., 2000). It has also been reported that butyric acid may play a role in the prevention and treatment of cancer (Scheppach et al., 1995), ulcerative colitis (Cummings, 1997) and human cancer cells (Whitehead et al. 1986; Gamet et al., 1992).

However, all these benefits to human health from the consumption of food containing oligosaccharides come with some discomfort as a result of hydrogen, carbon dioxide and methane production in addition to the short chain fatty acids. These gases produce flatus, cause diarrhea and abdominal pain (Reddy et al., 1980; Fleming, 1981) and for this reason these oligosaccharides are also considered anti- nutritional factors. The degree of this discomfort however, depends on the amount of oligosaccharides consumed and individual sensitivity to the intake of these alpha galactosides (Calloway et al., 1971; Price et al., 1988; Roberfroid et al., 1998). The production of discomfort and flatulence limits the use of lentil as food in human diet and is one of the main reasons behind low consumption of lentils around the world. For increasing lentil consumption as functional food and a source of good quality protein it might be helpful to reduce lentil oligosaccharides content to an acceptable level and to select lentil genotypes that will produce low oligosaccharide over a wide range of environments.

Although the content of various carbohydrates in seeds of many legumes has been reported, however, there is no systematic study reporting the carbohydrate profiles in legume seeds. Environment has a major effect on the accumulation of seed storage compounds, thus influencing the legume seed quality and its

utilization for food, feed or industrial applications. The present study aims to address some of these questions and was conducted with the following objectives.

OBJECTIVES

The long term objective of our research is to improve seed quality of grain legumes of interest to Saskatchewan producers. The current project was focused on lentils and had the following objectives:

(i) Develop carbohydrate profiles for Saskatchewan cultivars of lentils;
(ii) Study the effect of genotype x environment (GxE) interaction on the accumulation of selected carbohydrate(s);
(iii) Characterize the natural variation in selected carbohydrates present in global lentil germplasm;
(iv) Identify and characterize key enzymes participating in selected carbohydrate biosynthesis.

The results will be presented and discussed under each objective. Common biochemical methods will be presented in the Materials and Methods section. Plant material and experimental design is objective specific, therefore it will be presented under each objective.

C - MATERIALS AND METHODS

Sample Preparation: Prior to analysis the seeds were milled in an Udy cyclone mill to pass through a 0.5-mm screen. After milling in the UDY mill, all samples had a similar moisture content.

Protein Concentration: The protein concentration of chickpea seed samples was determined using the combustion method [AACC method 46-30 (AACC 2000)], using a FP-528 protein/nitrogen analyzer (LECO, St. Joseph, MI). The ground lentil sample was passed through hot copper to remove oxygen and to convert NOx into N2, followed by Lecosorb and Anhydrone treatment to remove carbon dioxide and water. The nitrogen in the sample is measured by the thermal conductivity cells (FP-528 Protein/Nitrogen Analyzer, Leco Corporation, St Joseph, MI, USA). The crude protein concentration of each sample was calculated using the following formula:
Crude protein (%) = % N x 6.25

Total Starch Concentration: Total starch content was determined using AACC method 76-13 (AACC 2000), using a commercial kit Megazyme (AA/AMG). 100 mg samples (UDY ground 0.5 mm) were suspended in 0.2 mL 80% (v/v) EtOH to aid dispersion and vortexed. Samples were incubated at 100°C for 8 min and vortexed every 2 min with α-amylase (AA, 300 units in MOPS buffer, 50mM, pH 7.0) to hydrolyze starch. Samples were incubated again at 50°C for 30 min with sodium acetate buffer (4 mL, 20 mM, pH 4.5) and amyloglucosidase (AMG, 0.1 mL, 20U) to hydrolyze dextrins to glucose. The content was diluted to 100 mL and 1 mL aliquot added to 3 mL glucose determination reagent (GOPOD) and incubated at 50°C for 20 min. TS was calculated as free glucose read at 510 nm absorbance as described.19 Commercial corn starch (Arancia Corn Products S.A de C.V, Mexico, D.F.) was used as a standard.

Isolation of starch granules: Starch was isolated from lentil seeds using a procedure described (Demeke et al, 1997). The seeds were cut into several small pieces and placed in 1 mL of sterile distilled water in a microfuge tube (1.5 mL) kept overnight at 4°C. The water was carefully decanted and a small pestle was used to grind the seed into slurry. The slurry was layered over 1 mL of cesium chloride (80% w/v) in a 2 mL microfuge tube. The microfuge tube was centrifuged at 13,000 g for 5 min. The supernatant was discarded and the starch granules pelleted at the bottom were centrifuged once again through cesium chloride to remove any adhering non-starch molecules. The pelleted starch granules were washed twice with buffer (55 mM Tris-HCl, pH 6.8, 2.3% (w/v) SDS, 10% v/v glycerol), once with water and finally with acetone. After the final wash, the starch granules were dried and used for amylose concentration determination.

Amylose determination: A high performance size-exclusion chromatography (HP-SEC) method was used to determine amylose concentration (Demeke et al., 1999). Five mg starch sample was suspended in 5 mL distilled water in a glass

tube and incubated at 130°C for 30 min. To 1 mL of gelatinized starch solution, 55 uL of 1M sodium acetate (pH 4.0) was added and mixed vigorously. Four units of isoamylase were added to de-branch the starch After 4 hours of incubation at 40°C, the debranching reaction was stopped by boiling for 20 min to inactivate isoamylase. The debranched starch solution was freeze dried. The freeze-dried sample was dissolved in 200 µL of DMSO (99% v/v) and then centrifuged in a microfuge at 15,000 g. Supernatant (40 uL) was injected in to a PLgel MiniMix-C guard column attached to a PLgel MiniMix 4.6 mm i.d. column (Polymer Laboratories, Inc. Amherst, MA) to separate amylose and amylopectin using a HPLC system (Waters 600 controller, Waters 610 fluid unit, Waters 717 plus autosampler, Waters 410 differential refractometer). The data were collected and analyzed using Millenium software (Waters HPLC). Starch samples, column, and detector were maintained at 40, 100 and 45°C, respectively. DMSO (99% v/v) was used as an eluent at a flow rate of 0.2 mL min-1. The amylose concentration was calculated by integration of the peak area corresponding to amylose to that of the peak area corresponding to both amylose and amylopectin.

Total soluble Sugars:

The concentrations of soluble carbohydrates of lentil seed meals were determined using an enzyme based method to hydrolyze sugars to glucose followed by colorimetric measurement of glucose using a raffinose/glucose assay kit (Catalogue no: K-RAFL, Megazyme International Ireland Ltd, Bray, Ireland). Soluble sugars such as RFO and sucrose were hydrolyzed with -galactosidase (from Aspergillus niger) and invertase (from yeast) into D-glucose, D-galactose and D-fructose. D-glucose concentration was determined using glucose oxidase peroxidase reagent (GOPOD reagent, Megazyme International Ireland Ltd, Bray, Ireland). The concentrations of raffinose, stachyose, verbascose and other higher homologues of the RFO in seed samples were measured as a group, because α-galactosidase hydrolyzes all members of the RFO family. Lentil seed meal (0.5±0.01g) was weighed into glass test tubes and 5 ml of 95% (v/v) ethanol was added to each tube. The lentil seed meal and ethanol mixture in the tubes was incubated in a water bath for 5 min (85^0C). After incubation the contents of each tube were quantitatively transferred to a 50 ml volumetric flask and the volume was made up with sodium acetate buffer (50 mM, pH 4.5). The contents of the volumetric flasks were mixed thoroughly for 15 min. After 15 min incubation 5 ml of the slurry was transferred to a glass tube, chloroform (2 ml) was added and the tube was centrifuged at 1000 g for 10 min. Supernatant (0.2 ml) of each sample was added to 0.2 ml of sodium acetate buffer (50 mM, pH 4.5), 0.2 ml of invertase (8.3U/ml) and invertase plus α--galactosidase enzyme solution (invertase 8U/ml and α--galactosidase 40U/ml) in separate glass tubes. All solutions were incubated for 20 min at 50^0C. GOPOD reagent (3 ml) was added to all the tubes including the reagent blank (0.4 ml sodium acetate buffer) and the glucose standard (D-glucose 100µg/0.1 ml) and all tubes were incubated for 20 min at 50^0C. The glucose concentrations of the samples were determined by the absorbance of quinoneimine dye at 510 nm using a spectrophotometer (DU 800, Beckman Coulter Inc). Glucose, sucrose and RFO concentrations of the lentil

samples are reported in millimoles /100 g lentil meal. The concentrations of soluble sugars were calculated as follows:
Glucose = $\Delta A \times F \times 50$,
Sucrose = $(\Delta B - \Delta A) \times F \times 50$,
RFO = $(\Delta C - \Delta B) \times F \times 50$,
'F' is a factor to convert absorbance to µmoles of glucose and ΔA, ΔB and ΔC are the absorbances of sample plus sodium acetate buffer, sample plus invertase and sample plus invertase and α--galactocidase enzyme solution respectively.

Extraction of RFO for composition analysis: Lentil seeds soluble carbohydrates were extracted with 80% (v/v) ethanol based on methods of Frias et al. (1994), Karoutis et al. (1992) and Sanchez –Meta et al. (1998) with some modifications. Seed meal (0.5 g) was intensively mixed with 10 ml of ethanol (80%, v/v) for 30 sec and incubated in a shaking water bath (Precision Scientific, Chicago, Illinois) for 45 min at 60°C with vigorous mixing at 15 min intervals. The solution was then centrifuged at 12,100 g for 10 min and the supernatant was collected. The sample pellet was re-extracted with 10 ml of ethanol (80%, v/v) as previously described and the two supernatants were pooled. The extract (5 ml) was passed through a C18 (Solid Phase System Columns, Honeywell Burdick & Jackson™) cartridge using a vacuum manifold to remove proteins and lipids present in the extract (Honeywell Burdick & Jackson™). The final 2 ml extract was collected and an aliquot (1.6 ml) was freeze dried and used for composition analyses.

High Performance Liquid Chromatography (HPLC) separation of RFO: Lentil seed RFO were separated by HPLC-SEC using a Sugar- Pak 1 column linked to a refractive index detector. Freeze dried samples were dissolved in 500 µL of nanopure filtered (0.2µ) water and centrifuged at 15,700g for 10 min. After centrifugation the samples were passed through 0.45 µm nylon syringe filters (Phenomenex, Torrance, CA, USA) and injected using an autosampler into a Sugar- Pak 1 column (Ca^{2+} cation - exchange resins) maintained at 90^0C. Nano pure filtered water containing CaNa2 EDTA (0.0001 M) was used as the mobile phase at a flow rate 0.5 ml/min. Sample run time and delay until the next injection was 20 min for each sample. RFO peaks in lentil samples were determined by comparing to retention times of pure raffinose and stachyose (Sigma) and verbascose (Fluka) standards. The combined area of raffinose, stachyose and verbascose in a sample was used to calculate the total RFO concentration in lentil cultivars. The percent area under each RFO peak in a sample was used to calculate the relative concentration of each specific oligosaccharide.

Statistical analyses: Analyses of variance (ANOVA) and correlation analyses were conducted using SAS version 9 (SAS Institute Inc, Cary, N.C). Duncan multiple range tests were used to separate means and significance was accepted at $P \leq 0.05$. Broad sense heritability of soluble carbohydrates in lentil cultivars was estimated from the components of analyses of variance (Singh et al., 1993) as follows:

σ^2
h2 = σ^2G / { σ^2G + GE /l+ 2e/rl}, where σ^2G and σ^2GE represent cultivars and cultivars-by-environmental variances, while r and l represent replications and locations respectively.

Analyses of variance (ANOVA) were performed for the individual experiments and combined ANOVA was conducted. A random model was used. Repeatability for starch, amylose and protein content measurements were estimated from components of variance using the equation:

$$\text{Repeatability} = \sigma^2 g / (\sigma^2 g + \sigma^2 g \times y / n + \sigma^2 g / n r)$$

where $\sigma^2 g$ = component of variance due to genetic differences among cultivars, $\sigma^2 g \times y$ = variance due to genotype × year interaction, $\sigma^2 y$ = variance due to the environment, n = number of years, r = number of replicates. The term repeatability refers to the constancy of cultivar starch, amylose and protein concentrations across years and replicates.

(d) RESULTS

Objective 1: Develop carbohydrate profiles for Saskatchewan cultivars and selected lentils

The main objective of this milestone is to develop a precise and reproducible method to extract and determine carbohydrates, namely starch and amylose concentrations, and to accurately identify and quantify oligosaccharides from lentil flour.

Starch and amylose concentration:

Total starch was analyzed using an enzymatic-colorimetric method essentially as described before (Hucl and Chibbar, 1996), whereas amylose content was determined by high performance liquid chromatography - size exclusion chromatography (HPLC-SEC) of de-branched starch as described (Demeke et al., 1999). In my laboratory the two methods had been optimized using wheat and barley flour. The same methods were applicable to lentil flour also. Therefore starch and amylose determination methods did not need any optimization.

Hulled vs De-hulled lentils for study

In the first experiment, twenty-two lentil lines (Table 1) were used to study the effect of de-hulling on total starch concentration. The seeds were manually de-hulled, and the weight of hull was measured in each line. The hulls on average accounted for 6.5 to 9.9% of the total seed weight (Table 1). The total starch concentration in various lentil genotypes varied from 41 to 46% in the hulled seeds. On de-hulling, the starch concentration ranged from 48 to 53%. The increase varied from 2 to 10%, depending upon the lentil genotypes (Table 1). This is interesting as the hull accounted for much narrower variation in dry weight. CDC Richlea had the highest starch concentration in both hulled and dehulled samples. In most of the cases, the increased starch concentration in the dehulled samples corresponded to the weight lost due to hulls. In some cases such as 1048-8R, 1294M-23 and 964a-46, the increase in starch concentration does not correspond to the hull weight. Starch is mostly stored in the cotyledons and hull only contains complex non-starch polysaccharides, the results obtained were as expected. A few lines which deviated from the expected results are interesting and will be explored further in another project. The main conclusion from this experiment was that keeping in mind the labor intensive requirement of de-hulling and the vast number of samples to be analyzed in this project, we will use seeds with hull in all future experiments. However, some lines such as 1048-8R, 1294M-23, 964a-46 are very interesting and will be investigated regarding the hull composition.

Table 1: Effect of de-hulling on total starch concentration in lentil seeds.

Genotypes	Starch (%)			Hull (% of grain weight)
	Hulled	De-hulled	Increase in de-hulled	
1048-8R	46.0	49.7	3.7	8.3
1294M-23	45.1	47.4	2.3	8.6
2670B	43.1	48.2	5.1	7.5
964a-46	44.6	49.1	4.5	8.3
ESTON	41.5	51.2	9.7	9.9
ILL 7502	44.5	53.0	8.5	7.7
ILL 1704	43.7	48.8	5.1	8.8
ILL 4605(Precoz)	44.9	51.7	6.8	8.6
ILL 5588(Northfield)	45.0	52.1	7.1	9.1
ILL 6979	44.7	52.4	7.7	8.3
ILL 7537	42.9	49.8	7.0	8.1
ILL 8006-BM4	43.7	51.7	8.0	8.1
PI 339283	43.3	50.5	7.3	7.1
PI 468901	40.2	50.7	10.5	9.9
PI 345629	41.8	50.4	8.6	6.5
1156-2-17	43.8	51.9	8.1	7.7
ILL 5684	45.1	52.5	7.4	7.9
RICHLEA	46.1	53.7	7.6	8.1
ROBIN	42.7	51.1	8.5	8.7
INDIANHEAD	43.2	51.1	8.0	8.7
REDBERRY	42.6	52.0	9.4	8.3
PI 320937	43.2	50.4	7.2	8.0

Optimization of methods for RFO analyses:

The enzymatic-colorimetric methods available for oligosaccharide analysis only determine total oligosaccharides content. High Performance Size Exclusion Chromatography (HPSEC) was used for the analyses because it can identify and quantify the individual oligosaccharides. Oligosaccharide analysis by gas chromatography needs volatilization step prior to analysis, which can be surpassed by using HPSEC. However, a major optimization step is to develop a method for complete extraction of oligosaccharides. The following three methods were investigated.

1. Based on Frias et al (1994): lentil flour was refluxed twice for 15 min with 80% ethanol, followed by washing the meal with 80% ethanol and with water. The extract was freeze dried.

2. Based on Karoutis et al (1992): lentil flour was homogenized for 2 min with 80% ethanol (1:15 w/v) after which the slurry was centrifuged for 10 min at

8000×*g*. The pellet was re-extracted in a similar fashion. The soluble protein in the extract was precipitated using a saturated lead acetate solution. Further purification was done by filtration through a 8 µm hydrophobic membrane filter and treatment with saturated monopotassium phosphate. The extract was freeze-dried.

3. Based on Sanchez-Mata et al. (1998), lentil flour was vigorously mixed with 80% ethanol (1:20 w/v) followed by incubating at 60°C for 45 min in a shaking water-bath whereby the sample was mixed thoroughly by vortex every 15 min. The flour-ethanol slurry was centrifuged at 8000×*g* for 5 min and the pellet and supernatant were separated. The pellet was re-extracted with ethanol as described above and the supernatants were combined and freeze dried.
Prior to analysis the freeze-dried extracts were reconstituted in water by mixing thoroughly followed by centrifugation at 8000×*g* for 10 min. The supernatant was filtered through a *Sep-Pak* cartridge (Waters Inc.) to remove particles, fat and proteins.

The samples were analyzed by HPLC with refractive index (RI) detector using a *Sugar Pak* column (Waters Inc.) and pre-column, at 90°C with water as the eluent at a flow rate of 0.5 mL /min. Raffinose, stachyose, and verbascose (Sigma Chemicals, St Louis, USA) were used as standards to determine the retention time and peak area of the individual oligosaccharides. The retention time was used to identify the oligosaccharides in the samples, whereas the peak area is a measure for the oligosaccharide content.

Optimization of a method for RFO analyses:

The three methods described above were tried to develop a simple, accurate and reproducible protocol for oligosaccharide analyses in a large number of samples. Method three, adapted from Sanchez-Mata et al (1998) to extract oligosaccharides in combination with HPLC-SEC using a RI detector was found to separate most of the oligosaccharides and sugars of interest (Figure 1). Verbascose, stachyose and raffinose eluted at around 5, 6 and 7 minutes. The raffinose peak was well separated but verbascose and stachyose were close togerher. However, there was reasonable valley area to separate the two components. The peak area corresponding to different concentrations of the three RFO was calculated. The peak area directly corresponded to the respective RFO concentration (Figure 2). This confirmed our results presented in Figure 1, that the method can resolve the three RFO components. In addition we can use this technique to determine relative concentrations of the three types of RFO. This being the first project, we were only interested in relative concentrations rather than the absolute concentrations of the three RFO. Finally, the oligosaccharides extracted using the method 3 from the lentil flour showed the presence of oligosaccharides by comparing with the retention times of oligosaccharides standards (Figure 3). The main conclusion of this experiment

was that we have optimized an adequate method to study the relative RFO concentrations in lentil seeds.

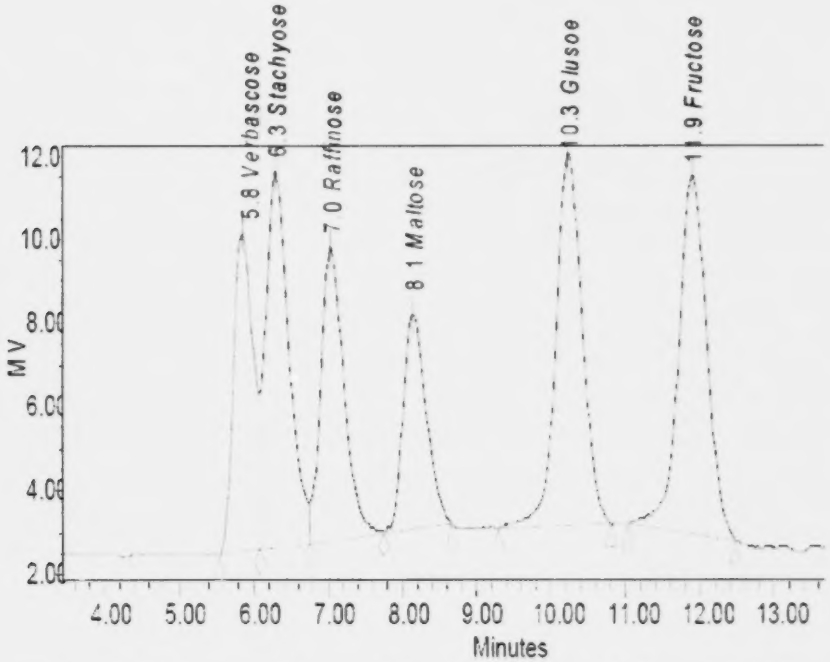


Figure 1. HPSEC separation profiles of a mixture of oligosaccharides and sugar standards to optimize the separation protocol.

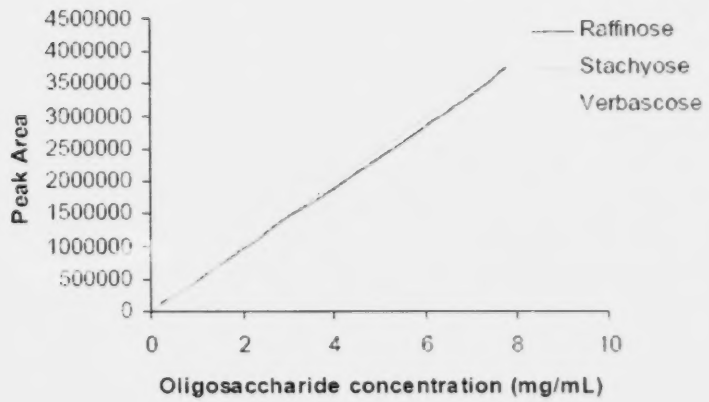


Figure 2: Standard curves to show that peak area corresponds to oligosaccharide concentrations. Peak area was used as a measure for oligosaccharide concentration.

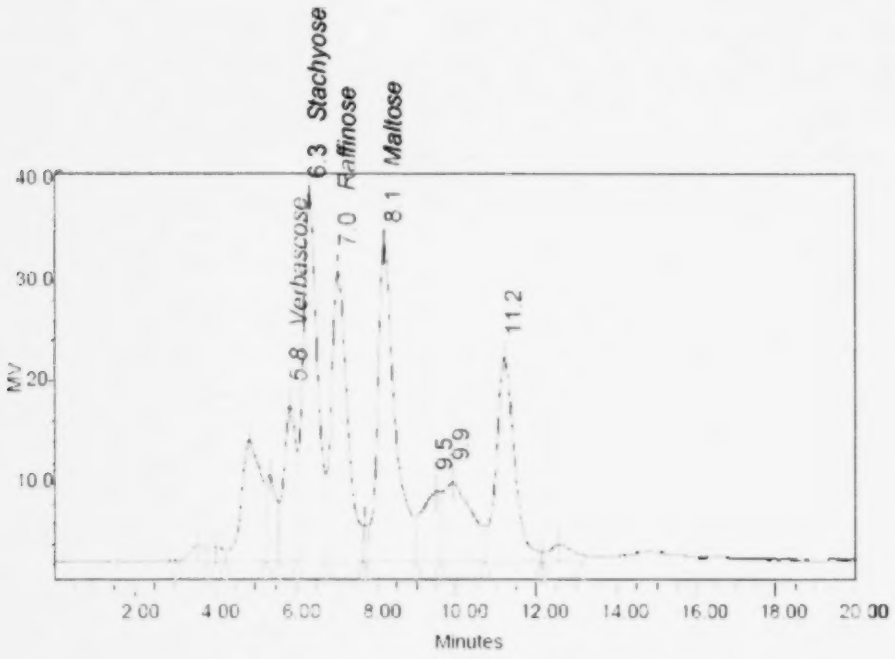


Figure 3: A representative HPSEC profile of ethanol extracts prepared from lentil flour to show the separation of three oligosaccharides and the presence of other soluble sugars, using the optimized protocol described in the text.

Carbohydrate profiles of selected lentil genotypes used in the CDC lentil improvement program:

Total starch and amylose concentration: The total starch concentration in the 22 lentil genotypes varied from 39 to 45% based on meal dry weight. Lentil line PI 468901 had the lowest (39%) starch concentration while, CDC Richlea and a line 1048-8R had the highest (45%) starch concentration (Table 2). The amylose concentration in general was around 30 to 34% of starch in all the lentil genotypes (Table 2). As compared to other storage starches, the lentil seed amylose concentration is slightly higher than other plant storage starches (Chibbar et al., 2004). However, there is very limited variation of only 4% between all the genotypes studied (Table 2). Lentil line PI 345629 had the highest (34%) and ILL6979 had the lowest (29%) amylose concentration among the lines analyzed in this study. Most of the lentil lines studied had an amylose concentration of about 33% of the total starch.

Table 2: Carbohydrate profile of selected lentil genotypes – Starch and amylose concentration (%, ±Standard Deviation).

Genotype	Starch	Amylose
1048-8R	45.1±0.69	32.2±0.04
1294M-23	44.2±0.63	31.7±0.7
2670B	42.3±0.8	32.8±0.5
964a-46	43.7±0.6	31.4±0.4
Eston	40.7±0.4	31.8±0.03
ILL 7502	43.6±1.1	30.7±0.4
ILL 1704	42.8±1.1	33.1±0.5
ILL 4605 (Precoz)	44.1±1.0	33.1±0.5
ILL 5588 (Northfield)	44.1±1.5	31.2±0.2
ILL 6979	43.8±0.4	29.8±0.3
ILL 7537	42.0±0.5	33.5±0.5
ILL 8006-BM4	42.8±0.4	33.5±0.2
PI 339283	42.4±0.5	33.8±0.1
PI 468901	39.4±0.5	32.0±0.6
PI 345629	41.0±0.9	34.0±0.1
1156-2-17	43.0±0.9	31.2±0.3
ILL 5684	44.2±1.2	30.9±0.3
Richlea	45.2±0.6	31.9±0.5
Robin	41.8±0.2	33.1±0.4
Indian Head	42.3±0.8	33.5±0.1
Redberry	41.8±2.9	33.7±0.1
PI 320937	42.4±1.0	31.4±0.6

Soluble sugars and raffinose family oligosaccharides (RFO) profiles in lentil lines:

An enzymatic method based on colorimetric detection of solubilized sugars was used to determine glucose, sucrose and total oligosaccharides. Glucose is present in very small quantities in lentil seed meal (Table 3). Sucrose and oligosaccharides concentrations are present around ten-fold higher than glucose. Sucrose concentration varied from 2 to 7 mmoles per 100g seed meal. CDC Indianhead had the lowest (2 mmoles/100g seed meal) and ILL 7537 had the highest (7.1 mmoles/100g seed meal) sucrose concentration. The total oligosaccharide concentration had very narrow range of variation (4.5 to 6.5 mmoles/100g seed meal) (Table 3). One third of the lentil genotypes had around 6 mmoles/100g seed meal), and the rest of the lines had around 5 mmoles/100g seed meal of oligosaccharides. Interestingly, CDC Indianhead had the lowest concentration of glucose, sucrose and total oligosaccharides (Table 3).

Table 3: Carbohydrate profile of selected lentil genotypes –Total soluble sugars, glucose, sucrose and oligosaccharides (mmoles/100g seed meal, ±Standard Deviation).

Genotypes	Glucose	Sucrose	Oligosaccharides
1048-8R	0.66±0.27	6.3±0.2	5.58±0.94
1294M-23	0.78±0.6	5.3±0.03	6.26±0.18
2670B	1.38±1.03	5.81±0.66	5.44±0.01
964a-46	0.93±0.71	4.76±0.27	6.46±0.06
ESTON	1.15±0.94	5.72±0.64	6.35±0.12
ILL 7502	0.68±0.48	4.96±0.47	6.10±0.31
ILL 1704	0.63±0.48	5.01±0.31	5.24±0.22
ILL 4605(Precoz)	0.51±0.33	4.78±0.30	5.95±0.21
ILL 5588(Northfield)	0.61±0.47	4.37±0.30	5.89±0.13
ILL 6979	0.87±0.67	4.13±0.49	6.15±0.03
ILL 7537	0.53±0.36	7.13±0.16	5.20±0.14
ILL 8006-BM4	0.50±0.31	3.78±0.26	6.53±0.12
PI 339283	0.59±0.34	4.48±0.17	5.99±0.22
PI 468901	0.57±0.14	4.36±0.01	6.57±0.06
PI 345629	0.53±0.14	3.72±0.03	5.76±0.03
1156-2-17	0.61±0.30	4.98±0.17	5.48±0.47
ILL 5684	0.89±0.71	5.89±0.42	5.85±0.12
RICHLEA	0.79±0.58	5.89±0.42	5.85±0.12
ROBIN	0.81±0.64	3.93±0.33	5.77±0.34
INDIANHEAD	0.57±0.32	2.00±0.16	4.57±0.49
REDBERRY	0.73±0.59	5.58±0.43	5.58±0.10
PI 320937	0.41±0.30	2.80±0.08	5.88±0.04

HPSEC analysis revealed that in lentil seed meal, raffinose was the most abundant RFO, closely followed by stachyose and verbascose constituted one-fourth of raffinose or stachyoze concentrations. Raffinose concentration varied from 16 to 33% of total soluble sugars, with CDC Indianhead having the highest raffinose concentration (Table 4). Highest stachyose (20.8%) was observed in ILL1704, while 26740B had the lowest (14.2%). Verbascose concentration varied from 1.9% to 8.5% (PI 339283) in the seed meal of lentil lines studied. A lentil line 2670B had the lowest amount of all RFO members studied in this experiment. Lentil line 2670B will be of interest for biochemical and molecular studies to understand the RFO biosynthetic pathway.

These analyses reveal some interesting lines, such as CDC Indianhead, which has very low sucrose and total oligosaccharides (Table 3), but the highest raffinose concentration (Table 4). Line 2640B has the lowest amount of the three RFO members.

Table 4: Carbohydrate profile of selected lentil genotypes – raffinose, stachyose, verbascose and other unresolved soluble sugars (relative concentrations, based on HPLC peak area integration values).

Genotypes	Raffinose	Stachyose	Verbascose	Other soluble sugars
1048-8R	20.8	16.9	5.1	57.2
1294M-23	21.6	16.4	6.1	55.9
2670B	16.6	14.2	1.9	67.4
964a-46	19.9	15.7	4.6	59.8
ESTON	18.2	17.6	3.7	60.5
ILL 7502	19.7	19.2	6.0	44.8
ILL 1704	22.4	20.8	5.4	51.4
ILL 4605(Precoz)	18.8	20.1	6.5	54.6
ILL 5588(Northfield)	20.9	17.6	4.8	56.4
ILL 6979	21.1	17.9	7.2	53.8
ILL 7537	ND	ND	ND	ND
ILL 8006-BM4	22.3	19.6	7.3	51.8
PI 339283	21.5	19.5	8.5	51.5
PI 468901	21.8	18.0	6.6	53.7
PI 345629	24.4	18.0	6.1	51.4
1156-2-17	21.6	14.1	4.1	60.2
ILL 5684	20.1	18.8	4.8	56.3
RICHLEA	24.4	18.9	4.8	51.9
ROBIN	26.7	19.3	4.6	49.4
INDIANHEAD	33.6	16.6	3.2	46.4
REDBERRY	22.5	16.1	4.4	57.1
PI 320937	26.8	19.5	5.3	48.4

Carbohydrate profiles in selected wild lentil species:

Very limited variation in starch and soluble carbohydrates was observed in the lentil genotypes being used in CDC lentil improvement programs. Therefore it was considered prudent to analyze starch and soluble carbohydrates with focus on RFO in selected wild lentil species. Six *Lens* species, *L. odemensis* (3 lines), L. tomentosus (3 lines), *L. lamottei* (2 lines), *L. ervoides* (5lines), *L. orientalis* (4 lines) and *L. nigricans* (5 lines), seeds were obtained and ground in to a meal. Some of these seeds are very small and were only available in very limited amounts. Starch and amylose concentrations were analyzed for all lines (Table 5), but RFO concentration analysis could not be completed for all lines (Table 6).

Starch and amylose concentration:

The total starch concentration in most of the wild lentil lines was lower than that observed in domesticated lines used in lentil improvement program (Tables 1 and 2). However, a much larger range of variation (33 to 42%) was observed among the wild lentil lines. Highest starch concentration (42%) was observed in a *Lens tomentosus* genotype, while lowest 33% was found in a *Lens nigricans* line (Table 5). The amylose concentration was very similar to those in the domesticated lentil lines (Tables 1, 2 and 5).

Table 5: Carbohydrate profile of selected wild lentil genotypes – Starch and amylose concentration (%, ±Standard Deviation).

Species	Accession PI #	Starch	Amylose
Lens odemensis	72746	36.1±1	29.0±0.86
	72688	34.7±0.04	32.0±0.09
	72745	38.6±0.03	30.1±0.04
Lens tomentosus	72614	39.6±0.05	31.9±0.17
	72830	39.6±0.26	29.2±0.06
	72643	42.3±0.16	29.8±0.05
Lens lamottei	110813	35.1±0.58	28.9±0.24
	110810	39.4±0.46	28.1±0.09
Lens ervoides	NEWL-2	39.2±0.49	31.2±0.37
	Lad # 28	40.1±0.59	29.5±0.48
	Lad # 81	41.3±0.85	29.5±0.25
	572335	38.0±0.61	32.4±0.83
	Lad # 32	40.9±0.82	31.5±0.04
Lens orientalis	NEWL-4	34.9±1.45	30.9±0.16
	72529	36.9±0.29	31.3±0.4
	72625	37.8±1.07	30.3±0.09
	72620	37.5±0.03	27.9±0.13
	Lazinisky # 22	38.6±0.71	28.5±0.40
Lens nigricans	Lad # 62	35.8±0.65	30.0±0.24
	572347	36.6±0.70	31.0±0.06
	Lad # 76	34.6±0.21	31.0±0.83
	Lad # 61	33.6±0.21	29.7±0.24
	57560	34.2±0.52	30.5±0.89

Raffinose family oligosaccharides (RFO)

A considerable amount of variation was observed in the RFO composition in seed meal of wild lentil species. In contrast to domesticated lentil lines, raffinose concentration did not significantly vary in wild lentil lines. In most lines studied raffinose concentration varied from 14 to 18% (Table 6) as compared to 16 to 33% in domesticated lentils (Table 4). In wild lentil lines stachyose concentration varied from 10 to 25% (Table 6) which is more than that observed in

Table 6: Carbohydrate profile of selected lentil genotypes – raffinose, stachyose, verbascose and other unresolved soluble sugars (relative concentrations, based on HPLC peak area integration values).

Species	Accession PI #	Raffinose	Stachyose	Verbascose	Other soluble sugars
Lens odemensis	72746	18.25	16.97	1.49	63.29
	72688	15.69	15.75	1.51	67.05
	72745	17.14	19.89	1.61	61.35
Lens tomentosus	72614	18.78	18.49	0.76	61.97
	72830	14.86	15.64	1.65	67.84
	72643	18.74	19.69	1.33	60.25
Lens lamottei	110813	16.68	12.78	2.87	67.67
	110810	16.95	10.31	2.47	70.28
Lens ervoides	NEWL-2	X	X	X	X
	Lad # 28	X	X	X	X
	Lad # 81	16.30	15.88	12.24	55.59
	572335	15.57	15.06	9.03	60.33
	Lad # 32	12.73	16.32	12.24	55.59
Lens orientalis	NEWL-4	18.36	25.48	1.59	54.57
	72529	17.85	19.90	1.07	61.18
	72625	18.59	21.59	1.23	58.59
	72620	X	X	X	X
	Lazinisky # 22	14.11	25.52	3.77	56.60
Lens nigricans	Lad # 62	X	X	X	X
	572347	X	X	X	X
	Lad # 76	X	X	X	X
	Lad # 61	X	X	X	X
	57560	X	X	X	X

domesticated lentils. *Lens orientalis* is interesting because it has stachyose as the dominant RFO, as compared to raffinose in all domesticated and wild lentils studied in this project. However, the most interesting results were obtained for verbascose, which varied from 1 to 12% in wild lentils (Table 6). *Lens ervoides* consistently had very high verbascose concentration (9-12%) as compared to other lentils (Table 6).

The main objective of this milestone was to develop reliable and reproducible methods to analyze starch and amylose concentrations and other soluble carbohydrates with a focus on RFO. The methods were successfully developed and used to develop carbohydrate profiles for 22 lentil lines currently used for lentil improvement at CDC. In addition, several wild lentil species were also analyzed.

Major conclusions from this study are that there is limited variation in starch and amylose concentration in lentil germplasm currently being used in lentil improvement program. Similar conclusions were also made for soluble carbohydrates and RFO members. The additional work with wild lentil species clearly showed that there is significantly more variation available in wild lentils. In this study limited number of lines was used. However, it strengthens the point to screen global germplasm to clearly identify material for further detailed biochemical and molecular studies.

Objective 2: Study the effect of genotype and environment (GxE interaction studies) on the accumulation of selected carbohydrate(s) in lentils.

Environmental factors affect the composition and quantity of carbohydrates in plants, as they do with other seeds storage components. A three-fold increase in stachyose concentration was observed in buckwheat seed embryos grown at low temperatures (18^0C) compared to embryos grown at higher temperatures (25^0C) (Horbowicz et al., 1998). Similarly, sucrose and RFO, particularly raffinose concentration, increased in Xerophyta viscose (family Vellociaceae) leaves under water deficit conditions (Peters et al., 2007). In this study we examined the genetic variability, heritability and effect of environment on the concentration and composition of lentil seed starch, amylose and soluble carbohydrates.

Material

Eleven lentil (*Lens culinaris* Medikus subsp. culinaris) cultivars (Table 7) were grown in a randomized complete block design with three replications in two environments in 2004 and eight environments in 2005 in Saskatchewan, Canada (Table 8). Each cultivar was grown in four rows 4.6 m long and 0.3 m apart under

Table 7: Lentil cutivars and their seed soluble carbohydrates concentration (mmole/100 g seed meal)

Cultivar	Cotyledon Color	Glucose	Sucrose	Total RFO
CDC Sovereign	Green	0.17	4.9	5.5
CDC Vantage	Green	0.32	3.7	5.4
CDC Glamis	Green	0.17	3.7	5.2
CDC Meteor	Green	0.14	3.6	5.2
CDC Richlea	Green	0.23	4.0	5.1
CDC Milestone	Green	0.18	4.4	4.9
CDC Viceroy	Green	0.14	4.1	4.8
CDC Rouleau	Red	0.12	4.6	5.3
CDC Robin	Red	0.13	3.5	4.8
CDC Rosetown	Red	0.10	3.8	4.6
CDC Redberry	Red	0.14	4.6	4.5
Mean		0.17	4.1	5.0
Range		0.10 - 0.32	3.5 - 4.9	4.5 - 5.5
Std Error		(±0.02)	(±0.1)	(±0.1)

Table 8: Soil type, and monthly precipitation (mm) in different environments

Environments	Soil Zone	May	June	July	Aug	Sep	Total
Kyle 2005	Brown	57.4	165.0	21.3	30.6	117.9	382.2
Rouleau 2005	Dark Brown	52.5	92.5	34.5	39.5	17.5	236.5
Rosthern 2005	Dark Brown	30.5	110.0	55.0	60.5	78.0	334.0
Swift Current 2005	Brown	29.0	96.5	27.0	49.0	50.0	251.5
Scott 2005	Dark Brown	50.7	88.2	60.4	71.5	93.5	364.3
Davidson 2005	Dark Brown	73.0	157.2	43.8	40.2	79.7	393.7
Davidson 2004	Dark Brown	20.0	58.0	57.0	119.4	22.5	276.9
Goodale 2004	Dark Brown	31.0	78.0	72.5	77.0	21.5	280.0
Goodale 2005	Dark Brown	30.5	110.0	55.0	60.5	78.0	334.0
SPG 2005	Dark Brown	30.5	110.0	55.0	60.5	78.0	334.0

Source (http://www.theweathernetwork.com/

rain-fed conditions with no fertilizer application. The pre-emergence herbicide Imazethapyr was used (Pursuit, BASF Canada Inc.) to control weeds. The harvested lentil seed was cleaned and ground with a cyclone sample mill (Udy Corporation, Fort Collins, Colorado, USA). The meal was used to determine total soluble carbohydrate concentration and RFO compositional analyses.

Results

3.1. Effect of environment on lentil seed glucose and sucrose concentration

The glucose concentration of lentil cultivars ranged from 0.10 - 0.32 mmole/100 g seed meal. The mean glucose concentration for all selected cultivars was 0.17 mmole/100 g seed meal (Table7). Sucrose concentration of lentil cultivars ranged from 3.5 - 4.9 mmoles/100 g seed meal with a mean concentration of 4.1 mmole/100 g seed meal for all cultivars. The glucose and sucrose concentrations in lentil seed meal observed in this study concur with previously reported lentil seed concentrations of glucose (Bhatty and Christison, 1984) and sucrose (VidalValverde et al., 1993; VidalValverde and Frias, 1992).

The analysis of variance (Table 9) showed strong and significant (P≤0.0001) cultivar and environmental effects on glucose, sucrose and total RFO concentrations in lentil seeds. The effects of the interaction between cultivar and environmental conditions on soluble carbohydrates were also significant. Among cultivars CDC Vantage and CDC Richlea had significantly higher (P≤0.05) mean glucose concentrations of 0.32 mmole and 0.23 mmole/100 g lentil meal, respectively. CDC Rosetown (0.10 mmole/100 g lentil meal) had the lowest glucose concentration among all cultivars (Table 7). The glucose concentrations of lentil cultivars varied significantly over different environments. The mean glucose concentration of the lentil cultivars grown at Goodale 2004 (0.34 mmole/100 g lentil meal) was significantly (P≤0.05) higher than all other environments (Table 10). The lowest mean glucose concentrations were observed in lentil cultivars grown at Davidson 2004 (0.08 mmole/100 g seed meal). However, the glucose concentrations of lentil cultivars at this environment were not significantly different than glucose concentrations at Scott 2005 and Kyle 2005. Lentil seeds have very low glucose concentrations, which are very close to the detection levels of the method. In addition, at very low glucose concentrations, the color is not in the optimal range as per Beer-Lambert law for spectrophotometric detection. Therefore slight variation in color intensity can result in large CV, as noted in Table 9.

Table 9: Analyses of variance and variance components of seed soluble carbohydrates of lentil cultivars

Source	Df	Mean square		
		Total RFO	Sucrose	Glucose
Total	325			
Environments	9	1.9**	18.4**	0.2**
Environments*Replication	20	0.2 ns	0.2 ns	0.0**
Cultivar	10	3.0**	6.4**	0.1**
Cultivar*Environments	90	0.4**	0.7**	0.1**
Residual	196	0.2	0.2	0.0
$\sigma^2_{Environmental}$		0.0 (± 0.0)	0.6 (±0.3)	
$\sigma^2_{Cultivar}$		0.1 (±0.1)	0.2 (±0.1)	
$\sigma^2_{Cultivar\ s\times Environment}$		0.1 (±0.0)	0.2 (±0.0)	
$\sigma^2_{Residual}$		0.2 (±0.0)	0.2 (±0.0)	
CV		9.5	11.2	50.9
R-square		0.7	0.9	0.9

h^2	0.85	0.89

**, ns=Significant at 1% and not significant respectively

3.2 Lentil seed total RFO concentration

The total RFO content of lentil cultivars ranged from 4.5 - 5.5 mmole/100 g seed meal with a mean concentration of 5.0 mmole/100 g seed meal (Table 7). Comparison of the total lentil RFO concentration in this study with those in previous studies is difficult because the raffinose/glucose assay method measures total RFO as a group in mmoles instead of quantifying the concentrations of RFO individually in grams. The α-galactosidase mediated hydrolysis does not differentiate among individual RFO members and hence the concentration is representative of all oligosaccharides hydrolysed by the enzyme. Methods used previously for RFO determination are semi-quantitative and time consuming. Although the use of a specific method depends on the objective of the study, the colorimetric method has distinct advantages over other RFO measurement methods. Liquid and gas chromatographic methods rely on the use of expensive sugar standards, which sometimes are not available for all higher RFO homologues. The colorimetric method is less time consuming, quantitative and can be used for analyzing large numbers of samples.

Table 10 :Soluble carbohydrates (mmoles/100g seed meal) of lentil cultivars across different environments

Locations	Glucose	Sucrose	Total RFO
Goodale 2004	0.34	4.8	5.4
Rouleau 2005	0.20	3.6	5.3
Scott 2005	0.11	4.6	5.1
Swift Current 2005	0.20	3.0	5.1
Goodale 2005	0.17	4.3	5.1
SPG 2005	0.16	4.4	5.1
Rosthern 2005	0.20	5.5	5.0
Davidson 2005	0.13	3.7	4.9
Davidson 2004	0.08	3.6	4.7
Kyle 2005	0.12	3.4	4.6
Mean	0.17	4.2	5.0
Range	0.11 - 0.33	3.0 - 5.5	4.6 - 5.4

Std Error	(±0.0)	(±0.1)	(±0.1)

The variation in RFO concentrations of the selected lentil cultivars though statistically significant (P≤0.05), were not large (Table 7). This may be due to the fact that these cultivars share a common genetic background. Duncan's multiple range test, however, showed two distinct groups of lentil cultivars with contrasting RFO concentrations. The first group comprised of high RFO and included two sub groups. The first sub group is comprised of CDC Sovereign, CDC Vantage and CDC Rouleau, whereas the second sub group consisted of CDC Glamis, CDC Meteor and CDC Richlea. CDC Sovereign had a significantly higher (P≤0.05) RFO concentration than other cultivars in this group. The second group consisted of cultivars with the lowest mean RFO and included CDC Redberry, CDC Rosetown, CDC Viceroy, CDC Robin and CDC Milestone. In this group of cultivars CDC Redberry had the lowest RFO concentration of all cultivars followed by CDC Rosetown, although the two were not significantly different.

3.3. RFO composition of lentil seeds

HPLC-SEC analysis of lentil cultivars for RFO indicated significant cultivar, environmental and cultivar × environmental interaction effects on RFO composition in lentils (Table 11). Stachyose was the major RFO in all lentil cultivars followed by raffinose and verbascose (Table 12). Individual RFO composition of lentil cultivars remained the same in all environments (Table 13). Together, stachyose, raffinose and verbascose, made up 39.5 - 44.5% of the free sugars of lentil cultivars, which is similar to 38.0% reported earlier (Fleming 1981). The total of the stachyose, raffinose, and verbascose content of CDC Robin was significantly higher (P≤0.05) than all the other cultivars (Table 6). CDC Redberry on the other hand had the lowest total concentration of the three oligosaccharides. It may be interesting to note that RFO quantification results by the colorimetric method also indicated CDC Redberry had the lowest RFO concentration. However, it is difficult to compare the results of two methods for all cultivars, because for composition analysis only three RFO were considered in this study. The concentrations of any higher RFO homologues, hydrolyzed by α-galactosidase enzyme to determine the total RFO concentration in the colorimetric method were not accounted for in the compositional analysis by HPLC method.

The raffinose proportion of the combined total of the three oligosaccharides of lentil cultivars ranged from 39.1 – 44.6% (Table 12). CDC Rosetown raffinose concentration of 44.6% was significantly higher (P≤0.05) than all other cultivars except CDC Redberry with a raffinose concentration of 43.2%. CDC Sovereign raffinose concentration of 39.1% was significantly lower than the raffinose concentration of all cultivars except CDC Viceroy and CDC Milestone with raffinose concentrations of 39.2 and 40.5% respectively. The stachyose and verbascose concentrations of lentil cultivars ranged from 48.6 – 53.0% and 4.4 – 10.5%, respectively. CDC Rouleau had a significantly higher (P≤0.05) stachyose and a significantly lower (P≤0.05) verbascose concentration than all other cultivars. Across different environments the total concentration of these three

RFO together ranged from 37.0 - 44.9% of the total soluble sugars, and the concentrations of raffinose, stachyose and verbascose of ranged from 36.2 – 46.1%, 47.6 – 52.1 % and 6.0 – 12.7 % respectively (Table 13).

Table 11: Analyses of variance of three selected seed RFO members in lentil cultivars

Source	Df	Mean Square			
		Raffinose	Stachyose	Varbascose	Total RFO
Total	323				
Environments	9	307.6**	57.0**	143.4**	254.7**
Environments*Replication	20	11.7ns	7.0ns	2.6ns	4.5ns
Cultivar	10	80.5**	46.6**	100.7**	61.8**
Cultivar *Environments	90	12.6*	11.7**	4.4**	19.0**
Residual	194	8.4	7.7	1.5	5.5
CV		7.0	5.6	14.5	5.6
R-square		0.8	0.6	0.9	0.8

*, **, ns = Significant at P 0.01, < 0.01 and not significant difference, respectively

Table 12 : Concentration (%) of three selected seed RFO members in lentil cultivars.

Cultivar	Cotyledon	Raffinose	Stachyose	Verbascose	Total OS
CDC Meteor	Green	41.1	49.7	9.3	43.0
CDC Vantage	Green	42.6	48.7	8.7	42.7
CDC Viceroy	Green	39.2	50.3	10.5	42.7
CDC Glamis	Green	41.3	49.0	9.7	41.6
CDC Milestone	Green	40.5	50.5	8.9	41.1
CDC Sovereign	Green	39.1	50.7	10.2	40.6
CDC Richlea	Green	41.3	49.1	9.6	40.3
CDC Robin	Red	40.7	50.7	8.5	44.5
CDC Rosetown	Red	44.6	49.6	5.7	41.7
CDC Rouleau	Red	42.6	53.0	4.4	40.5
CDC Redberry	Red	43.2	48.6	8.2	39.5
Mean		41.5	50.0	8.5	41.7
Range		39.1 – 44.6	48.6 –53.0	4.4 – 10.5	39.5 - 44.5
Std Error		(±0.5-0.6)	(±0.5)	(±0.2)	(±0.4)

Table 13: Concentration (%) of three selected seed RFO members in lentil cultivars in different environments

Environments	Total oligosaccharides	Raffinose	Stachyose	Verbascose
Davidson 2004	44.9	42.4	48.8	8.9
Goodale 2004	44.6	36.2	51.1	12.7
Swift Current 2005	43.9	44.2	49.8	6.0
Davidson 2005	43.7	42.0	49.9	8.1
Rouleau 2005	43.0	43.7	49.5	6.9
Kyle 2005	42.3	46.1	47.6	6.4
Scott 2005	39.6	41.7	50.8	7.5
SPG 2005	38.9	38.2	52.1	9.7
Goodale 2005	38.8	37.9	51.1	11.0
Rosthern 2005	37.0	42.5	49.3	8.2
Mean	41.7	41.5	50.0	8.5
Range	37.0 - 44.9	36.2 – 46.1	47.6 – 52.1	6.0 – 12.7
Std Error	(±0.4– 0.4)	(±0.5 - 0.5)	(±0.5 – 0.5)	(±0.2 – 0.2)

3.4 Environment influences RFO concentration in lentil genotypes

The differences in the RFO concentrations among cultivars may be due to the difference in RFO biosynthetic enzymes, specifically their affinity for substrates. A comparative study of the enzymes of the RFO biosynthetic pathway in lentil genotypes with varying RFO concentrations has not been reported to date. The total RFO concentration of lentil cultivars varied significantly over different environments (Tables 10 and 11). Based on Duncan's multiple range test, environments can be divided into two different groups. The first group is composed of environments where high RFO concentrations were produced and consists of Goodale 2004 & 2005, Rouleau 2005, Swift Current 2005, Scott 2005, SPG 2005 and Rosthern 2005. Among these environments Goodale 2004 had the highest RFO concentration (5.4 mmole/100 g seed meal) followed by Rouleau 2005 (5.3 mmole/100 g seed meal), however, the RFO concentrations produced in these two environments were not significantly ($P \leq 0.05$) different. The second group of environments with lower RFO concentrations included Kyle 2005 and Davidson 2004 & 2005 with the lowest mean RFO concentration of 4.6, 4.7 and 4.9 mmole/ 100 g, respectively.

The environments included in this study differed in total precipitation received during the growing season, soil type and temperature (Table 8). The significant differences in RFO concentrations in lentil cultivars grown in different environments may be attributed to these variables. Environments in the high RFO group received comparatively lower total precipitation during the lentil growing season than the low RFO group. Rouleau 2005 received 236.5 mm of precipitation which was the lowest of all environments, whereas Goodale 2004 received precipitation of 280 mm, though higher than the Swift Current 2005 and Davidson 2004 precipitation of 251.5 mm and 276.9 mm, respectively, was considerably lower than the precipitation received in other environments. Davidson 2005 and Kyle 2005 environments constituting the low RFO group received the highest precipitation of 393.7 mm and 382.2 mm respectively, of all the environments. These results show that lentil cultivars generally produced higher seed RFO in environments that received less precipitation compared to environments that received more precipitation. These results indicate that soil moisture content and resulting water stress influence the RFO concentration in lentil seeds. Similar trends were not observed between a specific soil zone and RFO concentration in lentil cultivars. All high RFO group environments except Swift Current 2005 fall in the Dark Brown soil zone, whereas three environments i.e. Kyle 2005 and Davidson 2004 & 2005, forming the low RFO group belonged to the Brown and Dark brown soil zones, respectively. The Brown soil zone is characterized by higher temperatures, low soil organic matter and low moisture compared to Dark Brown zone. No conclusive correlation between soil zone and RFO concentration was determined. Although no major study has previously been conducted on the effect of different environmental conditions on the RFO concentration in lentils, abiotic stresses such as temperature and drought have been reported to affect the concentrations of soluble sugars, including RFO, in seeds of other legume plants (Piotrowicz-Cieslak, 2006) . The concentration and composition of RFO and sucrose in yellow lupin (Lupinus luteus L. cv. Juno) seeds varied with changes in temperature. Yellow lupin seeds that matured at high temperatures had more raffinose than seeds that matured at optimum temperatures and produced more stachyose. Seeds maturing at sub - optimal temperatures also contained high concentrations of sucrose and myo-inositol (Piotrowicz-Cieslak, 2006). Similarly, drought (Piotrowicz-Cieskak et al., 2007) and cold stress (Gilmour et al., 2000) have been reported to affect RFO biosynthesis in plants.

The significant environmental effect on the RFO production in lentil seeds raises the question of the feasibility of a future lentil improvement program. Although the variations in the concentration of RFO and other soluble carbohydrates in lentil cultivars due to differences in the soil and environmental conditions are statistically significant, they were not large and may not be significant with respect to the objective of lentil improvement for RFO concentration modification. The broad sense heritability (h^2) of RFO and sucrose calculated from the components of variance (Table 9) was 0.85 and 0.89, respectively. This suggests that selection of lentil cultivars and breeding lines with low RFO concentration might be possible. Correlation analyses showed a non-significant correlation

between glucose and sucrose (Table 14). Significant positive correlations between glucose and RFO and sucrose and RFO were found. These positive correlations between RFO and glucose and especially sucrose are expected as sucrose is a substrate for the RFO biosynthesis. These results are in agreement with a previous study (Karner et al., 2004), where they reported a moderate correlation between sucrose and RFO production in pea genotypes. CDC Sovereign's highest sucrose and total RFO concentrations indicate this important relationship between RFO and sucrose concentration.

Table 14: Correlation coefficients among lentil seed soluble carbohydrates.

	Glucose	Sucrose	Total RFO
Glucose		-0.1ns	0.3**
Sucrose			0.3**

**, ns=Significant at $P < 0.01$ and not significant, respectively.

Conclusions

Soluble sugars of lentil cultivars varied significantly and were affected by different environmental conditions resulting in cross-over interaction. A strong positive correlation between sucrose concentration and RFO concentration was observed. An inverse relationship between the total precipitation received during the lentil growing season at different growing environments and the RFO concentrations of lentil cultivars was also observed. Changes in RFO concentration due to environmental effects, though statistically significant, were small from a RFO improvement perspective. High heritability of RFO would allow exploitation of natural variation in domesticated and wild genotypes for lentil quality improvement.

Objective # 3: Screen the lentil global germplasm for variation in selected seed carbohydrates.

Land races of the cultivated lentil and wild species of lentil are a potential source of natural variation in RFO concentration. The genus *Lens* comprises four species and includes *L. culinaris* Medikus, *L. ervoides* (Brign.) Grande, *L. nigricans* (M. Bieb.) Godron, and *L. lamottei* Czefr. Sub species of are represented by *L. culinaris* subsp. *culinaris*, subsp. *orientalis* (Boiss.) Ponert, subsp. *tomentosus* (Ladiz) subsp. *odemensis* (Ladiz.) (Sarker and Erskine (2005). Any natural variation found in the RFO concentration in these species and sub-species may help in the development of cultivated lentil with reduced RFO concentrations.

Although the concentration and composition of RFO in lentil have been reported in few studies, in most cases these studies are based on a few varieties of lentil. The International Centre for Agriculture in Dry Areas (ICARDA) lentil collection has been evaluated for certain agronomic characters, early maturity and drought stress, however, no attempt has so far been made to evaluate this collection for variation in RFO concentration. Analyzing and screening of such a large germplasm collection (10,509 accessions) for a specific trait is not practical and therefore subsets (i.e. core and mini-core collections) drawn from the germplasm collection are analyzed to facilitate screening. A core collection is a chosen subset of large germplasm collection that generally contains about 10% of the total accessions and represents the genetic variability of entire germplasm collection. It captures most of available genetic diversity of the species (Brown 1989). The number of accessions in a core collection for many crops is in several thousands and therefore a subset of the core collection i.e. mini core, is drawn. The mini core consists of only about 1% of the entire collection but still represents the diversity of the entire core collection. The objective of this present study was to evaluate the extent and level of variation of RFO in ICARDA lentil mini core collection with the long term objective to develop cultivated lentil with reduced RFO concentrations.

Material

ICARDA lentil mini core collection comprising 122 domesticated and 46 wild accessions were grown in two environments in 2006 and in 2007 in Saskatchewan, Canada. The description of the various accessions is given in Tables 15 and 16. The harvested seeds were ground and the seed meal was analysed for RFO, sucrose and glucose concentrations as described in the Methods section.

Table 15: Names and country of origins of domesticated lentil (*Lens culinaris* subsp. *culinaris*) accessions

Crop Nr.	IG	Country of origin	Province	Crop Nr.	IG	Country of origin	Province
ILL 213	213	AFG	Farah	ILL 1553	1553	IRN	Esfahan
ILL 1762	1762	AFG	Paravan	ILL 2372	2372	IRN	
ILL 2217	2217	AFG		ILL 7621	107419	IRN	East Azerbaijan
ILL 7567	76286	AFG	Heart	ILL 4903	4903	IRN	Gilan
ILL 4605	4605	ARG		ILL 55	55	IRQ	Dahuk
ILL 86	86	ARM	Artik	ILL 5418	5418	ITA	Puglia
ILL 1671	1671	AZE	Nachichevan	ILL 9	9	JOR	Karak
ILL 7773	114558	BGD	Gopalganj district	ILL 5584	5584	JOR	Karak
ILL 5399	5399	BGR		ILL 6920	73784	JOR	Ma'an
ILL 6967	73831	BRA		ILL 5209	5209	JOR	Irbid
ILL 4349	4349	CAN		ILL 5883	69492	JOR	
ILL 2290	2290	CHL		ILL 1139	1139	LBN	
ILL 5490	5490	CHL		ILL 4804	4804	LBY	
ILL 7499	76218	CHN		ILL 6505	71456	MAR	Centre Nord
ILL 1649	1649	COL		ILL 7727	111991	MAR	Centre
ILL 5968	69577	CYP	Limassol	ILL 5425	5425	MEX	
ILL 4783	4783	CZE		ILL 624	624	MKD	Macedonia
ILL 4831	4831	DEU		ILL 4609	4609	NLD	
ILL 7051	73915	DZA	Batna	ILL 4782	4782	NOR	
ILL 784	784	EGY		ILL 3487	3487	NPL	Janakpur
ILL 6540	71491	EGY	Marsa Matruh	ILL 7314	76033	NPL	Janakpur
ILL 4387	4387	EGY	El Giza	ILL 7791	114842	NPL	Narayani
ILL 80	80	ESP	Castilla la Mancha	ILL 229	229	PAK	NWFP
ILL 5058	5058	ESP	Castilla la Mancha	ILL 2194	2194	PAK	Northern Areas
ILL 1683	1683	ETH	Shewa	ILL 6378	71310	PAK	NWF
ILL 1744	1744	ETH	Shewa	ILL 313	313	PAL	Haifa
ILL 1983	1983	ETH		ILL 5424	5424	POL	Lublin
ILL 2433	2433	ETH	Sidama	ILL 4956	4956	PRT	
ILL 5945	69554	ETH	Gonder	ILL 4774	4774	ROM	
ILL 4740	4740	FRA	Center	ILL 2191	2191	RUS	
ILL 293	293	GRC	Ionian Islands	ILL 7089	73953	RUS	Tambov oblast
ILL 4865	4865	GRC	Peloponnesus	ILL 7745	113079	SAU	Al-Baha, Aseer
ILL 494	494	GTM		ILL 5576	5576	SCG	Sebia

ILL 4915	4915	HRV	Croatia	ILL 1861	1861	SDN	Al Khartum
ILL 4665	4665	HUN		ILL 4785	4785	SVK	
ILL 132	132	IND	Uttar Pradesh	ILL 28	28	SYR	Homs
ILL 2501	2501	IND		ILL 5511	5511	SYR	Al Hasakah
ILL 2526	2526	IND		ILL 6689	71640	SYR	Al Qunaytirah
ILL 2581	2581	IND		ILL 6853	73717	SYR	Lattakia
ILL 2607	2607	IND		ILL 7747	114393	SYR	Aleppo
ILL 2684	2684	IND		ILL 4542	4542	SYR	Al Hasakah
ILL 2789	2789	IND		ILL 4400	4400	SYR	
ILL 3025	3025	IND		ILL 618	618	TJK	
ILL 3167	3167	IND		ILL 6182	70237	TUN	
ILL 3347	3347	IND		ILL 141	141	TUR	Tunceli
ILL 3596	3596	IND		ILL 572	572	TUR	Tunceli
ILL 3597	3597	IND		ILL 927	927	TUR	Kastamonu
ILL 3714	3714	IND		ILL 2171	2171	TUR	Hakkari
ILL 3805	3805	IND		ILL 7585	109044	TUR	Nigde
ILL 3925	3925	IND		ILL 6166	70221	TUR	Corum
ILL 4080	4080	IND		ILL 595	595	UK	Kiev
ILL 4164	4164	IND		ILL 3502	3502	UNK	
ILL 4359	4359	IND		ILL 4778	4778	URY	
ILL 5080	5080	IND	West Bengal	ILL 4671	4671	USA	
ILL 5151	5151	IND	West Bengal	ILL 4875	4875	UZB	
ILL 242	242	IRN	Fars	ILL 4768	4768	YEM	San'a
ILL 871	871	IRN	Mazandaran	ILL 2230	2230	YUG	
ILL 1048	1048	IRN	Fars	ILL 9901	138010		
ILL 1220	1220	IRN	Fars	ILL 10021	138130		
ILL 1337	1337	IRN	Khorasan	ILL 6264	71196		
ILL 1462	1462	IRN	Kerman	ILL 5845	69454		

Table 16: Names and country of origins of wild lentil genotypes

Crop_nr	IG	Latin name	Country of origin	Province
ILWL 20	72543	*Lens culinaris* subsp. *odemensis*	PAL	
ILWL 174	72697	*Lens culinaris* subsp. *odemensis*	SYR	Sweida
ILWL 202	72725	*Lens culinaris* subsp. *odemensis*	TUR	Van
ILWL 4	72527	*Lens culinaris* subsp. *orientalis*	TUR	K. Maras
ILWL 70	72593	*Lens culinaris* subsp. *orientalis*	IRN	Kerman
ILWL 73	72596	*Lens culinaris* subsp. *orientalis*	CYP	Nicosia
ILWL 212	72735	*Lens culinaris* subsp. *orientalis*	UZB	Kashkadar'ya
ILWL 330	72853	*Lens culinaris* subsp. *orientalis*	SYR	Aleppo

ILWL 335	72858	*Lens culinaris* subsp. *orientalis*	JOR	Balqa
ILWL 380	72903	*Lens culinaris* subsp. *orientalis*	TKM	Ashkhabad
ILWL 381	72904	*Lens culinaris* subsp. *orientalis*	TJK	Dushanbe
ILWL 91	72614	*Lens culinaris* subsp. *tomentosus*	TUR	Diyarbakir
ILWL 149	72672	*Lens culinaris* subsp. *tomentosus*	SYR	Aleppo
ILWL 42	72565	*Lens ervoides*	ITA	Puglia
ILWL 44	72567	*Lens ervoides*	SVN	
ILWL 51	72574	*Lens ervoides*	SCG	Montenegro
ILWL 52	72575	*Lens ervoides*	HRV	
ILWL 55	72578	*Lens ervoides*	PAL	Hefa
ILWL 62	72585	*Lens ervoides*	TUR	Antalya
ILWL 133	72656	*Lens ervoides*	SYR	Lattakia
ILWL 155	72678	*Lens ervoides*	SYR	Idlib
ILWL 262	72785	*Lens ervoides*	TUR	Antalya
ILWL 285	72808	*Lens ervoides*	TUR	K. Maras
ILWL 298	72821	*Lens ervoides*	SYR	Idlib
ILWL 328	72851	*Lens ervoides*	UKR	Respublika Krym
ILWL 339	72862	*Lens ervoides*	JOR	Irbid
ILWL 394	72917	*Lens ervoides*	LBN	Chouf
ILWL 412	107440	*Lens ervoides*	SYR	Lattakia
ILWL 461	116033	*Lens ervoides*	TUR	Izmir
ILWL 0	136624	*Lens ervoides*	YUG	
ILWL 0	136625	*Lens ervoides*	BIH	Boznia-Herzegovina
ILWL 0	140884	*Lens ervoides*	ARM	Syunik
ILWL 0	140929	*Lens ervoides*	AZE	Nagorna-Karabakh
ILWL 14	72537	*Lens lamottei*	FRA	Provence-Alpes-Cote d'
ILWL 432	110813	*Lens lamottei*	ESP	Andalucia
ILWL 437	116009	*Lens lamottei*	TUR	Aydin
ILWL 16	72539	*Lens nigricans*	FRA	Provence-Alpes-Cote d'
ILWL 112	72635	*Lens nigricans*	TUR	Antalya
ILWL 327	72850	*Lens nigricans*	UKR	Respublika Krym
ILWL 453	116025	*Lens nigricans*	TUR	Denizli
ILWL 474	116037	*Lens nigricans*	TUR	Manisa
ILWL 0	136636	*Lens nigricans*	ITA	Toscana
ILWL 0	136641	*Lens nigricans*	SCG	Montenegro
ILWL 0	136643	*Lens nigricans*	HRV	
ILWL 0	136645	*Lens nigricans*	BIH	Boznia-Herzegovina
ILWL 0	136648	*Lens nigricans*	ESP	Andalucia

RESULTS AND DISCUSSION

Soluble carbohydrates of domesticated lentil accessions (*Lens culinaris* subsp. *culinaris*) accessions:

The RFO and sucrose concentration and composition of individual oligosaccharides in domesticated lentil accessions is presented in Table 17. The RFO concentration of domesticated accessions ranged from 3.94-6.05 mmoles/ 100 g lentil meal. *Lens culinaris* accession ILL 1220 (Fars, Iran) had the highest mean RFO concentration whereas accession ILL 4778 (Uruguay) had the lowest mean RFO concentration. The sucrose concentration of lentil accessions ranged from 1.32-7.79 mmoles/100 g meal. Accession ILL 927 (Kastamonu, Turkey) had the highest sucrose concentration at 7.79 mmoles/100 g meal, and whereas accession ILL 4778 (Uruguay) had the lowest sucrose concentration at 1.32 mmoles/100 g meal. Differences in glucose concentration among domesticated accessions were small and ranged from 0.01-0.96 mmoles/100g meal. Accession ILL 4875 (Uzbekistan) had the highest glucose, whereas accession ILL1744 (Shewa, Ethiopia) had the lowest glucose concentration (Data not shown).

Considerable variation in the composition of RFO among domesticated lentil accessions was observed. Verbascose, raffinose and stachyose were present in all domesticated accessions. Stachyose was the major component of all domesticated lentil accessions except accession ILL 5424 (Lublin, Poland) where a slightly higher concentration of raffinose was observed. The verbascose concentration of RFO of domesticated lentil genotypes ranged from 3.7-13.8%. Accession ILL 6689 (Al Qunaytirah, Syria) had the highest concentration of 13.8 % whereas accession ILL 572 (Tunceli, Turkey) had the lowest verbascose concentration of 3.7%. The concentration of stachyose ranged from 45.6 - 58.4%. Accession ILL 1139 had the highest stachyose concentration of 58.4 % and originated from Lebanon. Accession ILL 6853 had the lowest stachyose concentration of 45.6% and originated from Lattakia, Syria. The raffinose concentration of the domesticated accessions ranged from 33.2-48.8 %. Accession ILL 1139 (from Lebanon) had the lowest raffinose content of 33.2 % while accession ILL 5424 had the highest concentration of 48.8 %.

Soluble carbohydrates concentration and composition of wild lentil accessions:

The RFO and sucrose concentration and composition of individual oligosaccharides in wild lentil accessions is presented in Table 3. Large variation in the RFO and sucrose concentration among wild lentil accessions was observed. Similarly large differences were observed in composition of individual RFO of wild lentil accessions. However, variation in glucose concentration among wild lentil accessions was not large (Data not shown). The sucrose concentration of wild accessions ranged from 0.44-4.88 mmoles/100g meal and was considerably lower than the domesticated lentil sucrose concentration (1.32-7.79 mmoles/100 g meal, Tables 17 and 18). *Lens ervoides* accession ILWL 42 (Italy) had the lowest sucrose concentration whereas *Lens culinaris* subsp *odomensis*

(ILWL 202) (Turkey) had the highest sucrose concentration. In general, accessions belonging to Lens ervoides had lower sucrose content (0.44-1.30 mmole/100g meal) than other wild lentil species. As mentioned above, the amount of glucose is very low as compared to sucrose and TRFO. The low amount of glucose results in very weak color development which does not follow the Beer-Lambert law of spectrophotometry, to draw meaningful conclusions. Therefore, we did not report the glucose concentrations for the minicore collection.

The TRFO concentration of wild lentils ranged from 1.71-5.28 mmoles/100 g meal. This RFO range is wider compared to domesticated lentil RFO range of (3.94-6.05 mmoles/ 100 g lentil meal, Table 17 and 18). The lowest TRFO concentration was observed in accession belonging to *Lens ervoides* (ILWL 285) originating from Turkey whereas the highest TRFO concentration was found in accession belonging to *Lens culinaris* subsp *odomensis* originating from Palestine. TRFO concentrations of accessions belonging to *Lens ervoides*, *Lens lamottei* and *Lens nigricans* were lower compared to the TRFO concentration of the wild subspecies of *Lens culinaris*.

More variation was observed in the concentration and composition of individual RFO in wild lentil compared to domesticated accessions. Large differences were also found in the individual RFO composition among wild species and subspecies of wild lentil. The concentration of verbascose ranged from 1.3-27.0 % of the total RFO concentration. The highest verbascose concentration of the TRFO was observed in *Lens ervoides* (ILWL 298) originating from Syria whereas the lowest verbascose concentration of the TRFO was found in *Lens culinaris* subspecies *orientalis* (ILWL 212) originating from Uzbekistan. Comparison of verbascose concentration of different wild lentil species showed that accessions belonging to *Lens ervoides* had higher verbascose concentrations compared to verbascose concentrations of *Lens culnaris* subspecies *orientalis*, *Lens culnaris* subspecies *tomentosus*, *Lens nigricans* and *Lens lamottei* accessions (Table18). The stachyose concentration of wild lentil accessions ranged from 31.8-56.2 %. The highest stachyose concentration was found in Len culinaris subsp. orientalis accession (ILWL 380) originating from Turkmenistan whereas the lowest stachyose concentration of the TRFO was found in *Lens nigricans* accession (ILWL 327) from Ukraine. The raffinose concentration of the TRFO ranged from 35.7-50.8%. The highest raffinose concentration was found in *Lens nigricans* accession (ILWL 327) from Ukraine, and the lowest in *Lens ervoides* accession (ILWL339) from Jordan.

Conclusions

Large variation was observed in soluble carbohydrates concentration and composition among domesticated and wild accessions. The sucrose and raffinose family oligosaccharides (RFO) concentration of domesticated lentil accessions ranged from 1.32 – 7.79 mmoles and 3.75 – 6.06 mmoles/100 g meal, respectively. Similarly, the concentration of RFO and sucrose in wild lentils

ranged from 1.71 – 5.28 mmoles/100 g, 0.44 – 4.88 mmoles/100 g, respectively. The individual RFO composition in domesticated lentils followed similar pattern. More variation in the composition of individual oligosaccharides was observed in the wild lentil accessions compared to domesticated accessions. Some wild lentil accessions had very low verbascose and higher concentration of raffinose than stachyose concentration. Variation observed in the concentration and composition of RFO in this study may be used in the development of low RFO lentil cultivars in the future.

Table 17: Mean soluble carbohydrates (glucose, sucrose and total RFO) concentration (mmoles/100 g lentil meal) and composition of RFO of domesticated lentil genotypes

Crop_nr	Sucrose	Total RFO	Ver	Stach	RAF
ILL 213	5.57±0.42	5.21±0.25	10.0±1.1	52.3±1.4	35.7±1.6
ILL 1762	4.64±0.30	5.11±0.18	8.2±1.1	53.8±1.4	39.0±1.6
ILL 7567	3.68±0.36	5.21±0.21	11.7±1.1	48.0±1.4	40.7±1.6
ILL 2217	4.16±0.30	5.39±0.18	4.5±1.1	51.7±1.4	43.1±1.6
ILL 4605	3.58±0.30	4.86±0.18	12.0±1.1	48.3±1.4	38.1±1.6
ILL 86	6.53±0.30	4.85±0.18	7.2±1.1	47.7±1.4	41.7±1.6
ILL 1671	5.38±0.30	5.41±0.18	8.1±1.1	52.7±1.4	37.1±1.6
ILL 7773	2.08±0.36	5.24±0.21	13.3±1.1	49.4±1.4	39.8±1.6
ILL 5399	1.88±0.36	4.97±0.21	10.5±1.1	52.1±1.4	39.4±1.6
ILL 6967	4.08±0.30	5.11±0.18	8.1±1.1	46.7±1.4	44.6±1.6
ILL 4349	2.97±0.30	5.20±0.18	11.5±1.1	49.3±1.4	39.7±1.6
ILL 5490	2.69±0.30	5.33±0.18	10.4±1.1	50.2±1.4	39.4±1.6
ILL 2290	3.07±0.36	5.15±0.21	8.1±1.1	48.9±1.4	42.6±1.6
ILL 7499	2.69±0.36	5.53±0.21	5.3±1.1	53.3±1.4	42.4±1.6
ILL 1649	3.87±0.36	5.64±0.21	5.7±1.1	55.7±1.4	38.5±1.6
ILL 5968	3.47±0.30	5.12±0.18	7.4±1.1	52.3±1.4	40.4±1.6
ILL 4783	2.98±0.30	5.38±0.18	8.0±1.1	52.1±1.4	40.1±1.6
ILL 4831	3.38±0.36	4.50±0.21	8.6±1.1	51.6±1.4	40.6±1.6
ILL 7051	4.22±0.30	5.16±0.18	12.1±1.1	47.1±1.4	39.6±1.6
ILL 784	2.71±0.36	5.33±0.21	11.4±1.1	53.5±1.4	36.2±1.6
ILL 6540	5.29±0.30	4.85±0.21	7.5±1.1	52.0±1.4	39.6±1.6
ILL 4387	4.44±0.30	5.35±0.18	7.5±1.1	51.6±1.4	40.4±1.6
ILL 80	3.26±0.30	5.05±0.18	10.9±1.1	49.5±1.4	38.2±1.6
ILL 5058	3.56±0.30	5.38±0.18	6.4±1.1	50.3±1.4	42.6±1.6
ILL 1744	2.41±0.36	5.60±0.21	12.0±1.1	54.6±1.4	34.9±1.6
ILL 1983	3.72±0.36	4.83±0.21	8.2±1.1	54.0±1.4	38.6±1.6
ILL 2433	2.14±0.30	5.08±0.18	10.3±1.1	51.7±1.4	39.7±1.6
ILL 1683	3.18±0.36	5.41±0.21	9.2±1.1	50.6±1.4	40.7±1.6
ILL 5945	2.92±0.36	5.01±0.21	10.0±1.1	49.4±1.4	41.7±1.6
ILL 4740	1.96±0.30	5.20±0.18	5.8±1.1	54.5±1.4	40.9±1.6
ILL 293	2.82±0.30	4.93±0.18	7.4±1.1	52.6±1.4	39.4±1.6
ILL 4865	2.78±0.30	4.46±0.18	8.4±1.1	50.9±1.4	41.0±1.6
ILL 494	2.62±0.30	4.93±0.18	8.4±1.1	50.9±1.4	39.6±1.6
ILL 4915	1.48±0.30	5.27±0.18	9.7±1.1	50.6±1.4	40.9±1.6
ILL 4665	3.57±0.30	5.27±0.18	9.1±1.1	49.8±1.4	41.1±1.6
ILL 2607	3.23±0.30	4.92±0.18	9.7±1.4	51.0±1.7	39.3±2.0
ILL 5080	1.92±0.36	5.61±0.21	11.7±1.1	51.6±1.4	39.5±1.6
ILL 5151	2.80±0.36	5.16±0.21	10.0±1.1	52.0±1.4	39.5±1.6
ILL 3167	3.32±0.36	5.21±0.21	9.3±1.4	52.0±1.7	40.3±2.0
ILL 4164	2.64±0.30	5.12±0.18	10.5±1.1	51.0±1.4	40.8±1.6
ILL 132	3.97±0.30	5.06±0.18	7.8±1.1	50.1±1.4	41.6±1.6
ILL 2789	3.49±0.36	4.60±0.21	8.9±1.1	48.9±1.4	41.7±1.6

ILL 4359	3.29±0.36	4.82±0.21	9.2±1.1	48.9±	41.8±1.6
ILL 3805	3.02±0.36	4.61±0.21	8.4±1.4	50.4±1.7	41.8±2.0
ILL 3925	2.97±0.36	4.83±0.21	7.6±1.1	50.5±1.4	42.1±1.6
ILL 3596	3.69±0.36	4.39±0.21	7.3±1.4	50.8±1.7	42.1±2.0
ILL 3714	3.04±0.30	4.94±0.18	6.0±1.4	51.7±1.7	42.1±2.0
ILL 3347	3.49±0.36	4.71±0.21	7.8±1.4	49.5±	42.4±2.0
ILL 2526	3.91±0.36	4.75±0.21	7.5±1.1	50.2±1.4	42.5±1.6
ILL 2684	3.40±0.36	4.52±0.21	8.3±1.1	48.6±1.4	42.6±1.6
ILL 2501	3.15±0.36	4.62±0.21	7.4±1.1	50.1±1.4	42.8±1.6
ILL 2581	3.55±0.30	4.69±0.18	7.8±1.1	49.3±1.4	43.0±1.6
ILL 3025	2.61±0.36	4.91±0.21	7.5±1.1	48.9±1.4	43.8±1.6
ILL 4080	3.29±0.36	4.67±0.21	7.5±1.1	48.2±1.4	44.1±1.6
ILL 3597	3.09±0.30	4.47±0.18	6.8±1.4	49.5±1.7	44.5±2.0
ILL 1337	4.93±0.30	5.57±0.18	10.9±1.1	52.9±1.4	35.5±1.6
ILL 1462	3.70±0.36	5.35±0.21	9.4±1.1	54.7±1.4	35.5±1.6
ILL 1553	5.97±0.36	5.06±0.21	8.1±1.4	54.8±1.7	37.2±2.0
ILL 871	5.49±0.36	5.43±0.21	10.0±1.1	50.2±1.4	37.4±1.6
ILL 4903	4.49±0.30	5.34±0.18	10.4±1.1	50.7±1.4	38.0±1.6
ILL 1048	4.42±0.30	5.81±0.18	4.7±1.1	55.5±1.4	38.6±1.6
ILL 242	4.10±0.30	5.24±0.18	7.6±1.1	52.0±1.4	39.0±1.6
ILL 7621	3.77±0.30	5.35±0.18	10.7±1.1	48.9±1.4	40.7±1.6
ILL 2372	4.43±0.36	4.90±0.21	7.9±1.1	48.7±1.4	41.8±1.6
ILL 1220	4.11±0.36	6.05±0.21	8.2±1.1	55.5±1.4	35.9±1.6
ILL 55	3.11±0.30	4.73±0.18	10.7±1.1	50.0±1.4	39.1±1.6
ILL 5418	2.68±0.30	4.81±0.18	10.3±1.1	48.4±1.4	42.4±1.6
ILL 9	3.79±0.36	5.24±0.21	9.9±1.1	51.7±1.4	38.4±1.6
ILL 5883	2.68±0.30	5.02±0.18	10.7±1.1	51.3±1.4	39.3±1.6
ILL 5584	3.61±0.36	5.19±0.21	12.0±1.1	49.9±1.4	40.0±1.6
ILL 5209	3.19±0.30	5.31±0.18	10.9±1.1	50.9±1.4	40.3±1.6
ILL 6920	3.18±0.30	4.96±0.18	10.0±1.1	48.5±1.4	43.6±1.6
ILL 1139	5.22±0.30	5.32±0.18	8.4±1.4	58.4±1.7	33.2±2.0
ILL 4804	3.05±0.30	4.93±0.18	9.0±1.1	52.4±1.4	38.6±1.6
ILL 7727	3.39±0.36	4.75±0.21	11.8±1.1	47.0±1.4	42.0±1.6
ILL 6505	3.29±0.36	4.81±0.21	7.4±1.1	47.0±1.4	44.6±1.6
ILL 5425	2.59±0.36	4.66±0.21	6.9±1.1	48.5±1.4	45.3±1.6
ILL 624	2.77±0.36	4.91±0.21	9.5±1.1	51.4±1.4	39.3±1.6
ILL 4609	2.77±0.36	4.73±0.21	10.8±1.1	46.7±1.4	41.6±1.6
ILL 4782	3.71±0.30	5.16±0.18	8.5±1.1	48.4±1.4	42.1±1.6
ILL 7314	3.38±0.36	5.90±0.21	5.8±1.1	52.6±1.4	41.2±1.6
ILL 3487	3.71±0.30	4.48±0.18	5.9±1.6	50.8±	43.2±2.3
ILL 7791	3.21±0.30	4.37±0.18	6.2±1.1	47.8±1.4	46.2±1.6
ILL 229	2.86±0.36	5.06±0.21	6.6±1.1	54.4±1.4	38.9±1.6
ILL 2194	4.17±0.30	5.75±0.18	5.4±1.1	52.8±1.4	41.4±1.6
ILL 6378	4.69±0.36	4.82±0.21	8.5±1.1	45.7±1.4	44.4±1.6
ILL 313	2.74±0.36	4.95±0.21	9.2±v	52.5±1.4	38.4±1.6

ILL 5424	1.76±0.36	4.54±0.21	6.2±1.1	46.1±1.4	48.8±1.6
ILL 4956	2.11±0.30	4.60±0.18	10.3±1.1	49.9±1.4	40.2±1.6
ILL 4774	3.10±0.30	4.78±0.18	7.5±1.1	50.6±1.4	41.7±1.6
ILL 2191	3.17±0.36	4.74±0.21	10.0±1.1	47.5±1.4	43.1±1.6
ILL 7089	2.59±0.36	4.25±0.21	9.9±1.1	46.1±1.4	44.7±1.6
ILL 7745	3.30±0.30	5.39±0.18	10.7±1.1	47.6±1.4	42.1±1.6
ILL 5576	3.58±0.36	4.57±0.21	8.7±1.1	47.6±1.4	42.0±1.6
ILL 1861	2.80±0.30	5.22±0.18	10.3±1.1	54.2±1.4	36.8±1.6
ILL 4785	3.03±0.30	4.28±0.18	9.9±1.1	47.7±1.4	42.8±1.6
ILL 6689	2.46±0.30	4.86±0.18	13.8±1.1	50.3±1.4	37.4±1.6
ILL 28	3.64±0.30	4.98±0.18	10.7±1.1	49.5±1.4	38.3±1.6
ILL 4400	3.81±0.30	5.27±0.18	11.6±1.1	46.0±1.4	41.4±1.6
ILL 4542	2.97±0.30	4.98±0.21	11.8±1.1	47.7±1.4	41.4±1.6
ILL 5511	2.50±0.30	4.88±0.18	10.8±1.1	47.7±1.4	42.0±1.6
ILL 7747	2.77±0.30	4.78±0.18	7.3±1.1	49.1±1.4	44.3±1.6
ILL 6853	3.84±0.36	4.91±0.21	8.9±1.1	45.6±1.4	45.0±1.6
ILL 618	4.84±0.30	4.85±0.18	9.5±1.1	48.3±1.4	39.3±1.6
ILL 6182	2.37±0.30	4.36±0.18	9.3±1.1	48.6±1.4	42.2±1.6
ILL 927	7.79±0.30	5.10±0.18	6.2±1.1	53.7±1.4	35.6±1.6
ILL 572	4.60±0.30	5.80±0.18	3.7±1.1	55.9±1.4	39.0±1.6
ILL 2171	2.85±0.30	4.89±0.18	10.8±1.1	51.2±1.4	39.3±1.6
ILL 141	4.28±0.30	4.75±0.21	6.7±1.1	51.7±1.4	40.7±1.6
ILL 7585	3.09±0.30	4.48±0.18	9.7±1.1	48.3±1.4	42.4±1.6
ILL 6166	3.99±0.30	5.22±0.18	5.6±1.1	51.3±1.4	42.8±1.6
ILL 595	3.49±0.36	5.12±0.21	9.9±1.1	52.6±1.4	37.3±1.6
ILL 3502	3.07±0.30	5.00±0.18	5.5±1.6	49.3±	43.7±2.3
ILL 4778	1.32±0.36	3.94±0.21	10.1±1.1	47.4±1.4	44.8±1.6
ILL 4671	2.38±0.36	5.10±0.21	11.3±1.1	[illegible]7±1.4	37.9±1.6
ILL 4875	3.53±0.30	5.11±0.18	9.6±1.1	51.6±1.4	38.2±1.6
ILL 4768	3.80±0.30	4.72±0.18	9.7±1.1	47.9±1.4	42.0±1.6
ILL 2230	2.91±0.30	5.07±0.18	7.7±1.1	52.8±1.4	41.5±1.6
ILL 10021	2.63±0.30	4.92±0.18	12.3±1.4	49.9±1.7	38.1±2.0
ILL 6264	3.21±0.30	5.08±0.18	10.9±1.1	50.4±1.4	39.4±1.6
ILL 9901	3.17±0.30	4.94±0.18	9.6±1.4	50.1±1.7	40.6±2.0
ILL 5845	2.72±0.30	5.30±0.18	10.9±1.1	47.7±1.4	42.3±1.6
Range	1.32-7.79	3.94-6.05	3.7-13.8	45.6-58.4	33.2-48.8

Table 18: Mean soluble carbohydrates (sucrose and total RFO) concentration (mmoles/100 g lentil meal) and composition of RFO of wild lentil accessions

Crop_nr	IG	Sucrose	Total RFO	Ver	Stach	Raf
***Lens culinaris*. subsp. odemensis**						
ILWL 20	72543	2.64±0.34	5.28±0.32	4.3±1.4	54.1±3.0	42.7±2.9
ILWL 174	72697	1.92±0.34	4.85±0.32	3.8±1.1	50.9±2.5	46.4±2.4
ILWL 202	72725	4.88±0.34	2.96±0.32	13.0±1.4	36.5±3.0	49.8±2.9
Range		1.92-4.88	4.85-5.28	3.8-13.0	36.5-54.1	42.7-49.8
***Lens culinaris*. subsp. orientalis**						
ILWL 4	72527	1.25±0.34	4.57±0.32	2.5±1.6	53.7±3.5	46.0±3.4
ILWL 70	72593	2.72±0.28	4.69±0.26	1.9±1.1	53.0±2.5	45.2±2.4
ILWL 73	72596	4.06±0.40	4.35±0.38	3.3±1.6	46.7±3.5	52.2±3.4
ILWL 212	72735	1.96±0.28	2.78±0.26	1.3±1.1	44.7±2.5	52.5±2.4
ILWL 330	72853	1.47±0.34	3.57±0.32	4.5±1.1	50.2±2.5	51.8±2.4
ILWL 335	72858	2.24±0.34	4.14±0.32	4.7±1.1	46.2±2.5	49.0±2.4
ILWL 380	72903	2.26±0.56	3.99±0.53	4.6±1.6	56.2±3.5	41.5±3.4
ILWL 381	72904	3.50±0.34	3.38±0.32	4.4±1.1	40.1±2.5	53.3±2.4
Range		1.25-4.06	2.78-4.69	1.3-4.7	40.1-56.2	41.5-53.3
***Lens culinaris*. subsp. tomentosus**						
ILWL 91	72614	1.31±0.28	4.11±0.32	2.0±1.1	43.5±2.5	54.5±2.4
ILWL 149	72672	1.01±0.28	3.69±0.26	4.2±1.1	47.7±2.5	52.6±2.4
Range		1.01-1.31	3.69-4.11	2.0-4.2	43.5-47.7	52.6-54.5
Lens ervoides						
ILWL 42	72565	0.44±0.34	2.13±0.32	22.5±1.6	39.1±3.5	40.7±3.4
ILWL 44	72567	0.67±0.34	2.19±0.32	25.9±1.4	38.2±3.0	37.0±2.9
ILWL 51	72574	1.13±0.40	3.19±0.38	23.6±1.6	40.0±3.5	38.6±3.4
ILWL 52	72575	1.02±0.34	2.85±0.32	22.3±1.6	36.4±3.5	43.5±3.4
ILWL 55	72578	0.64±0.28	2.39±0.26	23.7±1.4	39.1±3.0	38.4±2.9
ILWL 62	72585	0.88±0.40	2.96±0.38	24.4±1.6	40.9±3.5	36.9±3.4
ILWL 133	72656	0.80±0.40	2.89±0.38	24.1±1.4	38.4±3.0	40.4±2.9
ILWL 155	72678	0.92±0.34	2.62±0.32	25.8±1.1	37.9±2.5	38.5±2.4
ILWL 262	72785	0.54±0.34	2.53±0.32	24.9±1.1	33.7±2.5	43.2±2.4
ILWL 285	72808	0.61±0.34	1.71±0.32	23.9±1.6	33.7±3.5	43.4±3.4
ILWL 298	72821	0.82±0.28	2.84±0.26	27.0±1.4	37.8±3.0	39.8±2.9

ILWL 328	72851	0.80±0.40	2.02±0.37	24.7±2.2	36.9±5.0	41.9±4.8
ILWL 339	72862	1.27±0.28	3.04±0.26	26.1±1.1	39.7±2.5	35.7±2.4
ILWL 394	72917	1.30±0.40	3.42±0.37	22.5±1.4	41.0±3.0	39.3±2.9
ILWL 412	107440	0.50±0.34	1.90±0.32	20.2±1.1	34.4±2.5	48.8±2.4
ILWL 461	116033	1.30±0.40	2.92±0.37	23.3±1.4	36.7±3.0	42.2±2.9
ILWL 0	136624	0.94±0.56	2.93±0.53	25.0±2.2	36.7±5.0	39.2±4.8
ILWL 0	136625	0.72±0.56	2.77±0.37	23.0±1.6	34.7±3.5	44.4±3.4
ILWL 0	140884	0.90±0.34	2.77±0.32	14.9±1.4	37.0±3.0	50.8±2.9
ILWL 0	140929	0.70±0.28	2.09±0.26	22.4±1.1	37.8±2.5	41.3±2.4
Range		0.44-1.30	1.71-3.42	14.9-27.0	33.7-41.0	35.7-50.8
Lens lamottei						
ILWL 14	72537	1.26±0.34	2.19±0.32	11.2±1.6	37.1±3.5	53.9±3.4
ILWL 432	110813	1.44±0.28	2.48±0.26	7.0±1.1	37.5±2.5	50.6±2.4
ILWL 437	116009	1.72±0.34	3.16±0.32	5.6±1.1	40.7±2.5	58.9±2.4
Range		1.26-1.72	2.19-3.16	5.6-11.2	37.1-40.7	50.6-58.9
Lens nigricans						
ILWL 112	72635	2.40±0.40	3.41±0.37	2.1±1.4	42.5±3.0	56.5±2.9
ILWL 327	72850	1.17±0.40	2.40±0.37	2.3±1.4	31.8±3.0	70.0±2.9
ILWL 453	116025	1.28±0.34	3.40±0.32	4.7±1.4	44.7±3.0	59.0±2.9
ILWL 474	116037	2.00±0.34	3.02±0.32	4.3±1.4	37.0±3.0	60.9±2.9
ILWL 0	136636	1.07±0.34	2.57±0.32	3.0±1.4	36.0±3.0	67.4±2.9
ILWL 0	136641	1.71±0.28	2.49±0.26	3.2±1.1	37.2±2.5	63.6±2.4
ILWL 0	136643	0.78±0.28	2.49±0.26	3.3±1.4	38.5±3.0	62.1±2.9
ILWL 0	136645	0.92±0.34	2.73±0.32	5.6±1.4	36.5±3.0	62.4±2.9
ILWL 0	136648	0.92±0.40	3.32±0.37	2.2±1.4	38.8±3.0	65.3±2.9
Range		0.78-2.40	2.40-3.41	2.1-5.6	31.8-44.7	56.5-70.0
Range in all accessions		0.44-4.88	1.71-5.28	1.3-27.0	31.8-56.2	35.7-70.0

Objective 4: Biochemical analysis of the RFO biosynthetic pathway.

The first three objectives resulted in the RFO characterization (quantity and components) of lentil germplasm. The next step is to characterize key biosynthetic steps to develop molecular markers for use in marker assisted selection. RFO biosynthesis starts with the formation of a trisaccharide raffinose by the reversible addition of galactosyl units from galactinol (α-galactoside of myoinositol (O-α-D-gaactopyranosyl-1-(1→1)-L-myo-inositol) to sucrose. The synthesized trisaccharide, raffinose, serves as an acceptor for another galactosyl residue from galactinol giving rise to a tetrasaccharide stachyose (Peterbauer and Richter, 2001). These two reactions are catalyzed by raffinose synthase (enzyme code EC 2.4.1.82) and stachyose synthase (EC 2.4.1.67), respectively. A specific galactosyltransferase (EC 2.4.1.123) utilizes UDP-galactose and myoinositol to produce galactinol for raffinose oligosaccharides (Loewus and Murthy 2000). Biochemical analyses of a mutation in soybean that confers decereased RFO and phytic acid accumulation, suggests that the myoinositol supply for galactosyltransferase (EC 2.4.1.123) is a controlling factor determining the conversion of RFO (Hitz et al. 2002).

Therefore, we focused on Galctinol synthase (a galactosyltransferase; EC 2.4.1.123) for this part of our project. Galactinol synthase synthesizes RFO by catalyzing the formation of galactinol, (a galactocyle donor or galactose transfer intermediate) from UDP-gal and myo-inositol (Sarawitz et al., 1987; Lowell and Kuo, 1989; Keller, 1992). The production of galactinol is thought to be the first committed step in the biosynthesis of RFO and it also plays a key regulatory role in the carbon partitioning between sucrose and RFO (Saravitz et al., 1987). Galactinol may also function as scavenger/antioxidant leading to reduced cell oxidative damage and increased plant tolerance of environmental stresses (Nishizawa et al 2008).

The objective of the current study was to optimize conditions for GS enzymatic assay, and use the optimized method to investigate GS enzyme activity at various stages of lentil seed development. The ultimate objective is to determine the relationship of GS activity to lentil seed RFO (concentration and quality).

Materials and Methods

Six domesticated lentil accessions with contrasting RFO concentrations were selected from the ICARDA lentil mini core collection. The RFO concentration of the accessions is given in Table 19.
Growth conditions:

The selected accessions were grown in 2 gallon pots in a growth chamber (21^{o}C day and 15^{0}C night temperature; 18 hr light and 6 hr dark). Fertilizer (Nutricot 14-14-14) was applied to the plants, a week after emergence.

Table 19: Lentil lines selected for RFO biosynthetic enzyme assays.

Domesticated lentil accession #	Country of Origin	Mean total RFO seed (mmmoles/100g meal)
123	Iran	6.0
211	Nepal	6.0
196	Jordan	5.0
126	Iran	5.1
172	Uraguay	4.0
175	Slovekia	4.3

Developing lentil seeds were collected at 8, 16, 24, 32 and 40 days after flowering (DAF) (Fig 5a.) and immediately frozen in liquid nitrogen and stored at -80 0C until use. The seeds collected were used to determine the GS enzyme activity calorimetrically.

GS enzyme extraction:
Seeds (0.3 g) were ground in a mortar with 0.3 ml of cold extraction buffer (50 mM HEPES, pH 7.5, containing 1 mM DTT and 1 mM PMSF) for preparation of GS extract. The homogenate was centrifuged at 13,200 x g for 55 min at 4 0C and the supernatant (GS extract) was used for GS assays. The total protein concentration of the extract was determined by Brad ford dye binding assay.

Enzyme assay procedure:

To 50 µL of galactinol synthase extract was added 60 mM myo-inositol, 2 mM DTT, 50 mM HEPES buffer (pH 7.5), 4 mM MnCl2, 20 mg of bovine serum albumin and 4 mM UDP gal in a total volume of 100 µl. For control, the enzyme in the extract was killed by placing 50 µL of extract for 2 min in a boiling water bath. The enzyme reaction mixture was incubated for 30 min at 32ºC and terminated by heating reaction mixture in a boiling water-bath for 2 min.

After terminating the reaction 500 µL of water, 10 µL (0.3 U) of insect or potato apyrase solution, and 150 µL of apyrase reaction mixture (Tris-HCl buffer, 250 mM, pH 7.5, containing 25 mM KCl, 7.5 mM CaCl2, 0.5mM EDTA-Na, and 50 mM glucose) was added to the enzyme assay mixture. The reaction mixture was incubated for 10 min at 37ºC. After incubation the reaction was stopped by adding 60 µL of 75% tri-chloro acetic acid (TCA). The tubes were cooled on ice for 10 min, centrifuged at 3,000 x g for 10 min and the amount of Pi in the supernatant was determined by a modified Fiske and SubbaRow (1925) protocol as follows:

To each tube was added 100 µL of 2.5% ammonium molybdate dissolved in 2 N HCl and 40 µL of Fiske and SubbaRow reducer. The reaction was allowed for 2 min at room temperature. After 2 min, 40 µL of a 34% sodium citrate.$2H_2O$ solution was added to the tubes and the absorbance was measured at 660 nm. The amount of UDP formed by GS was determined by constructing a standard curve made with UDP hydrolyzed by the apyrase and by correlating it to the amount of UDP produced by galactinol synthase. GS activity was reported in µmol of UDP min-1mg-1 of total protein.

Results:

Optimization of GS enzyme assay:

Standard curve of UDP-hydrolysed by apyrase:

The colorimetric method for GS assay is based on the indirect determination of the UDP formed from myoinositol and UDP-gal by the action of GS. The UDP formed was hydrolyzed by exogenous apyrase and the resulting Pi quantified by a modification of the colorimetric method of Fiske and SubbaRow (1925). The amount of UDP formed by the GS was determined using a standard curve (Fig 2) constructed with UDP hydrolyzed by the apyrase, and correlated to the amount of UDP produced by the galactinol synthase. A very high correlation (r^2 = 0.9818) was observed between UDP concentration and inorganic phosphate released by apyrase (Fig. 4a). This suggested that inorganic phosphate can be used as a measure of UDP formed.

pH optimization for GS activity in lentil crude extract:

To determine the optimum pH for GS activity in developing lentil seeds, crude extracts prepared from CDC Redberry seeds (18 DAF) was assayed for GS activity at different pH (Fig. 4b). The GS enzymatic activity was lowest at pH 5, and gradually increased to reach highest at pH 7.5. Considerably low activity was observed at pH 8 (Fig 4b). Highest specific activity of 0.032 µmole UDP $min^{-1}mg^{-1}$ of protein was obtained at pH 7.5, therefore, subsequent GS activity analyses were out at pH 7.5. This pH falls with in the range of GS optimal pH range of 5.5-7.5 reported previously (Peterbaur and Richter 2001). Considerable activity was detected at a pH range of 5.0-8.0.

GS activity of lentil seed crud extract at different temperature:

GS enzymatic activity was lowest in enzyme assays conducted at 25°C, but it increased gradually till 50°C and decreased at 55°C (Fig. 4c). This decrease in activity at temperatures above 50°C is in agreement with results previously reported for soybean galactinol synthase (Ribeiro at al 2000) and indicate protein denaturation at temperatures above 50°C.

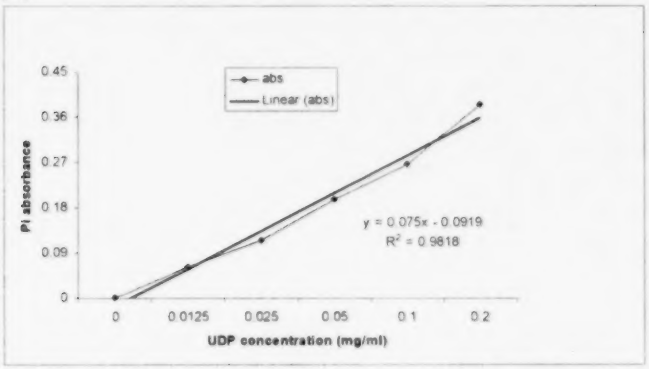
GS activity of lentil accessions with different RFO concentrations:

Initial results of the analysis of GS specific activity in the crude extracts of lentil seeds with three different RFO concentrations showed a similar trend. GS specific activities of all accessions were higher at 16 DAF and reduced considerably at 24 and 32 DAF, as the seed matured. The highest activity of 0.16 µmole UDP min-1mg-1 of protein was recorded for accession 126, followed by 0.14 µmole UDP min-1mg-1 of protein for accession 196 at 16 DAF (Fig 4). These accessions (126 and 196) belonged to medium RFO concentration of 5.1 and 5.0 mmole/100 g seed meal, respectively. The GS specific activity of the accession 172 was 0.09 µmole UDP min-1mg-1 of protein. The GS activity in lentil accessions 123 and 211 was 0.09 and 0.05 µmole UDP min-1mg-1 of protein, respectively (Fig 5b and c). The GS specific activity of 0.05 µmole UDP min-1mg-1 of protein of accession 211 was the lowest of all accession at 16 DAF. Accession 123 and 211 had the highest RFO concentration of all five accessions with 6.0 mmole/100 g seed meal. These results might suggest a lack of association between GS specific activity and RFO concentration in developing lentil seeds. However, total specific activity over all stages has yet to be determined to make a final conclusion in this regard. These GS specific activities in the crude extracts of lentils observed in this study are very similar to GS specific activities of 0.0055 µmole min-1mg-1 in the crude extracts of kidney bean and GS specific activity of 0.013 µmole min-1mg-1 in the crud extract of Zucchini leaf (Liu et al 1995).

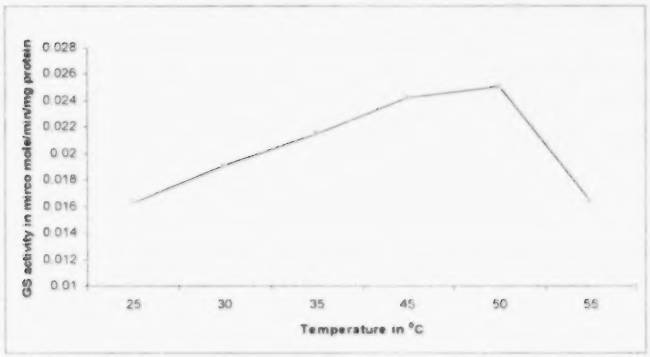
At 24 DAF no considerable difference in the GS activity of accessions 126, 196 and 172 was found. The GS activity of accession 126, 196 and 172 was 0.035, 0.029 and 0.031 µmole UDP min-1mg-1 of protein. Similarly, at 32 DAF GS activity in lentil seeds of all accessions was negligible with rates of 0.002, 0.008 and 0.016 µmole UDP min-1mg-1 for accession 126, 172 and 196, respectively. GS activity in developing soybean seeds has been reported to increase with the dry matter. Highest activity was reported for seeds with 42% of dry matter, corresponding to 6.5 reproductive growth stage (Fehr et al., 1971).

The results reported in this section are preliminary and more work is currently in progress.

(a)



(b)



(c)

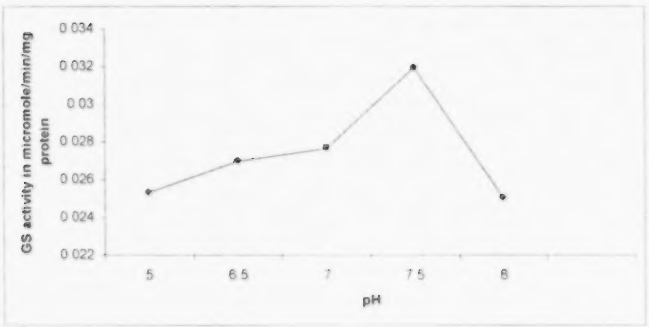
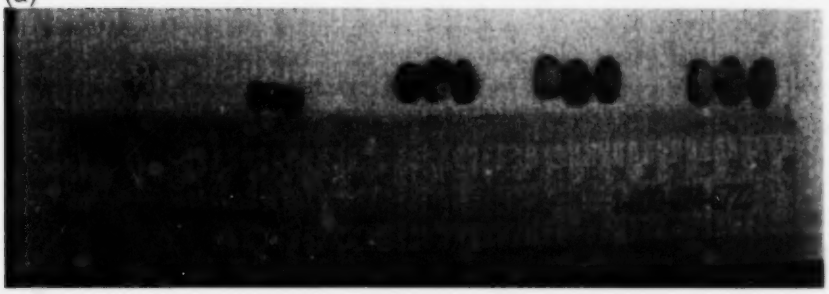
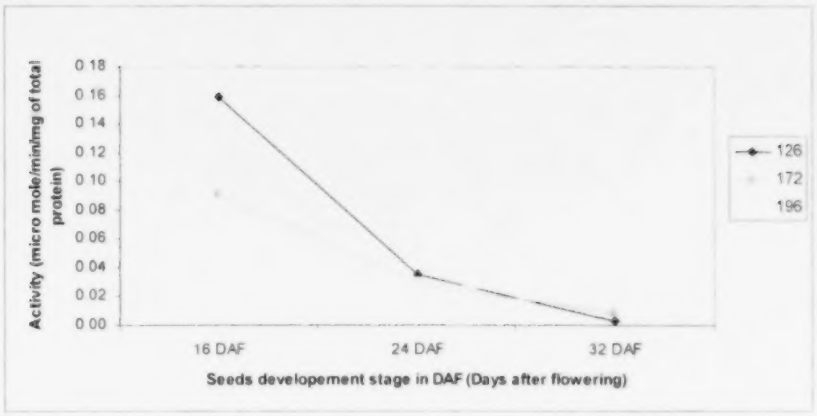


Figure 4: Optimization of galactinol synthase enzymatic activity. (a) Standard curve for inorganic phosphate production at different concentrations of UDP hydrolysis by apyrase. (b) GS activity at different temperatures of incubation, (c) GS activity at different pH of reaction mixture assay.

(a)



(b)



(c)

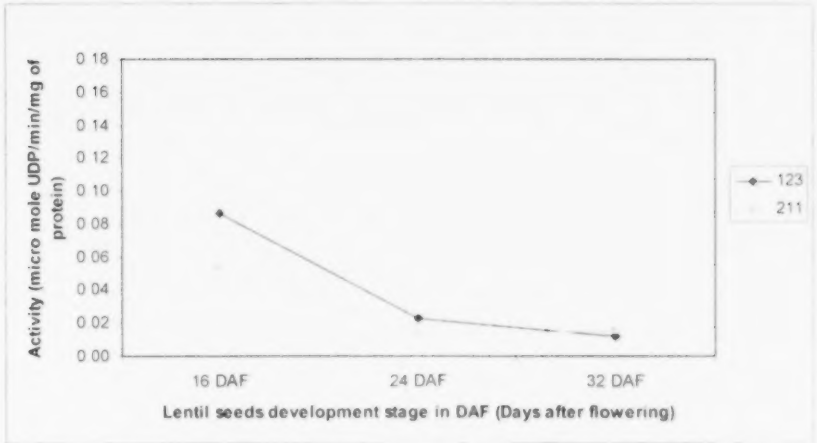


Figure 5: Galactinol synthase activity in lentil seeds at different stages after flowering (a) Lentil seeds at different days after flowering, 8, 16, 24, 32 and 40 DAF (left to right), (b) and (c) GS activity in seeds of different lentil lines with varying RFO concentrations.

(e) CONCLUSIONS AND RECOMMENDATIONS

This was the first systematic study focused on carbohydrates in lentils. The main conclusions from each milestone are summarized at the end of the respective objective. However, general conclusions are that there is less variation in carbohydrate concentration among the lentil varieties and germplasm currently in use in lentil improvement programs. Environment affects the accumulation and type of RFO in lentil seeds. However, RFO concentration is a highly heritable trait, thus standard plant breeding techniques can be used for lentil improvement. There is considerable amount of variation in wild lentil species and to some extent in global lentil germplasm collection. Therefore, standard plant breeding techniques can be used to develop lentil cultivars with reduced RFO concentration. However, considerable time and resources are needed to develop and test new varieties therefore, it will be advisable to develop molecular strategies to develop lentil varieties with changed carbohydrate profiles.

At present, there is lack of understanding how gene expression and carbohydrate seed content are related. Therefore, recently a new research project 'Understanding and improving lentil and chickpea seed quality – resduction of raffinose family oligosaccharides' under the NSERC-CRD in collaboration with Saskatchewan Pulse Growers (C$534,760) for a period of five years has been initiated as of March 02, 2009. The NSERC-CRD project was based on the results obtained in the recently completed ADF project. The successful completion of NSERC-CRD research proposal will develop a platform capable of relating the seed RFO concentration and composition of different lentil and chickpea varieties to their genetic composition. The goal is to develop targeted DNA-based molecular markers and genome maps to screen germplasm and expedite breeding for specific traits using marker assisted selection.

(f) ACKNOWLEDGEMENT

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(i) OTHER – (e.g. extension meetings: papers produced; conference presentations made, personnel involved; equipment bought; photos; project materials developed; expense statement).

Papers produced:

Tahir, M., Vandenberg, A. and Chibbar, R.N. (2009) Influence of environment on seed soluble carbohydrates in selected lentil cultivars. Journal of Food Composition and Analysis (submitted).

Conference Presentations:

Chibbar, R.N. (2009) Grain legumes for ensuring nutritional security. Plenary lecture – Nutritional security and human health- International Conference on Grain Legumes: Quality improvement, value addition and trade (ICGL 2009) Indian Institute of Pulses Research, Kanpur, India February 14-16, 2009.

Poster presentations:

Tahir, M., Lindeboom, N.,Verma, B., Båga, M., Vandenberg, A. and Chibbar, R.N. (2006) Seed oligosaccharide content and composition of selected lentil (Lens culinaris) genotypes. The thirteenth annual life & health sciences student research conference, University of Saskatchewan, Saskatoon, March 17, 2006. **(Second prize winner for poster award in the Plant Sciences theme).**

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Personnel involved

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